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THE RETICULOENDOTHELIAL SYSTEM (RES)*

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* This series of papers is the result of a conference on *The Reticuloendothelial System (RES)* held by The New York Academy of Sciences on December 10 and 11, 1959.

Part III The Relations of the Reticuloendothelial System to Infection and Immune Reactions

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Part I Definitions and Morphologic Aspects of the Reticuloendothelial System

REACTION PATTERNS OF THE RETICULOENDOTHELIAL SYSTEM UNDER STIMULATION*

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Scientists are still attempting to delineate the reticuloendothelial system (RES) anatomically and to determine its physiological properties.[1-10] These problems have not been solved because of its functional complexity and also because of a lack of experimental procedures that yield readily interpretable results. One method for carrying out delineation studies has been that of stimulating the system by any of a variety of methods and following the sequence of attendant histocytological changes. This method has not proved to be entirely satisfactory because it affects not only the RES proper but other cells and tissues as well, thereby giving rise to a complex of simultaneous variables. Nevertheless, this method should not be discarded out of hand until it can be superseded by one more clearly reliable or until a concerted effort has been made to minimize its inherent faults.

One popular method for stimulation of the RES is that of administering acid colloidal materials and following their accumulation within the macrophages. Such a method serves to locate phagocytes or phagocyticopotential elements when the colloidal particles are brought into association with these cells under conditions advantageous for ingestion. The selection of a colloid suitable for this purpose has proved to be difficult: some are rapidly eliminated from the body, others tend to flocculate in body fluid, and still others are markedly toxic to the host. *In the first series of studies to be reported here* a suspension of thorium dioxide (Thorotrast) has been used as a test colloid in rats and mice because of its relative stability, slow elimination, and, in low dosage, relative lack of toxicity.[11-[illegible]0] In a second series of experiments the same tissues and organs were studied in hosts bearing an actively growing Ehrlich ascites carcinoma. This was done in order to determine whether there are histological evidences of RES stimulation in cancer-bearing animals.

RE Responses Following Injection of Thorotrast

The reaction of the RES to intravenous Thorotrast is easily followed histologically by sacrifice of animals within a few days after even a single injection. The progressive activation of phagocyticopotential cells is followed by increasing the number of doses and the time interval before sacrifice.

Preliminary electron microscopic studies have been made to determine the essential features of phagocytosis. These studies indicate that in the Kupffer macrophages isolated particles of Thorotrast are taken in through cell membranes, particularly in the regions between microvilli. Such particles are at

* The work reported in this paper was supported in part by a grant from the National Institutes of Health, Public Health Service, Bethesda, Md.

first scattered at random throughout the macrophage cytoplasm and are secondarily collected into groups These groups are characterized by association with small areas of dense cytoplasm As greater numbers of particles become collected within a single group they are forced into association with each other to form small lattices and chains (FIGURE 1)

The injection of Thorotrast into subcutaneous tissues or other areas rich in loose connective tissue is followed by formation of a compact nodule rich in macrophages and infiltrated with collagenous and elastic fibers With increasing age these nodules become progressively smaller and firmer in texture (FIGURE 2a) Their more deeply placed macrophages undergo fusion to form multinucleate giant cells while the number of stromal fibers increases In rare instances (a total of 5 from our entire series of more than 4000 subcutaneous inoculations) neoplastic changes may appear within the period of 1 year Such nodules begin to enlarge grossly and, upon palpation become softer in consistency Histologically, they exhibit many elongated fibroblasts with rather chromatophilic nuclei (FIGURE 2b and c) None of these nodules proved to be transplantable

Liver After a single dose of Thorotrast a few hepatic macrophages can be identified by their collection of granules Following multiple doses almost every lining cell has become phagocytic and with still heavier doses, the cells are highly distended thereby distorting the sinusoidal pattern By using serial sections it can be shown that these loaded cells pull free from their connections in the sinusoidal walls At least some of these freed cells disintegrate, thereby liberating their store of globular clumps (FIGURE 2d)

Spleen Most of the Thorotrast particles in the spleen are taken up by free macrophages of the red pulp (FIGURE 2e) These cells appear in great numbers and are apparently derived by transformation of lymphocytes that migrate from the margins of the white pulp nodules After prolonged injection of Thorotrast the red pulp becomes filled with clusters of these loaded macrophages thereby distorting the pattern of the organ as a whole At the same time there is gradual depletion of white pulp so that the lymphoid component becomes small and inconspicuous

Adrenal After multiple spaced dosages of the colloid fixed macrophages become activated in the cortical and medullary sinusoids of the adrenal These macrophages are freed to collect in the lumens of sinusoids in the zona reticularis (FIGURE 2f)

Lymph nodes Lymph nodes are most readily stimulated by injection of Thorotrast into the peritoneal cavity from which it is drained principally by the lymphatics and filtered through the mesenteric and mediastinal nodes [11] In such organs both the bridging and littoral macrophages are readily stimulated to phagocytosis (FIGURE 3a) When the dosage is repeated and spaced over a considerable period cortical and medullary macrophage appear to have comparable avidity in collecting the particulate matter

Migrating lymphocytes are readily converted into macrophages within the lymph node where they typically appear as lightly loaded free cells in the sinusoid lumen They are readily differentiated from the heavily burdened phagocytes that were freed from the sinusoidal walls

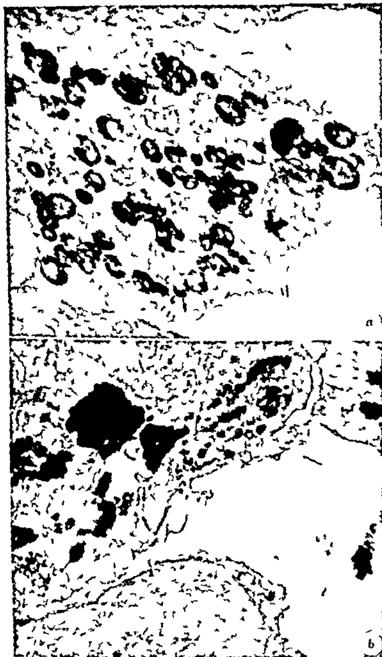


FIGURE 1 (a) Electron micrograph of a Kupffer macrophage with collections of Thorotrast This cell projects into the sinusoidal lumen shown at the right The Thorotrast particles appear to collect first at the surfaces of condensed areas of cytoplasm seen here intermixed with mitochondria The masses of phagocytosed material can be seen readily with a light microscope Microvilli appear as extensions of the cytoplasmic membrane at the lower right a small group of Thorotrast particles appears in the area between 2 microvilli This relationship is frequently seen and may represent the mechanism by which particles are taken into the cell. A small part of the Kupffer cell nucleus is seen at the extreme left ×10 350 (b) Electron micrograph of Kupffer cell macrophages nuclei are seen at top and bottom Note the foamy character of the cytoplasm and the minute Thorotrast particles scattered in areas near the collected masses ×10,350



FIGURE 2

Thymus It becomes evident that repeated injections of Thorotrast are toxic to the host as indicated by loss of appetite and weight as well as by the appearance of accidental involution of the thymus.[27] This latter phenomenon is not of precipitate nature and can be followed histologically by the gradual loss of thymocytes and an associated increase in the prominence of its stromal cell. As the thymocytes become depleted these stromal cells assume a rounded form and take on phagocytic properties. If injections of Thorotrast are continued the great majority of stromal elements collect and store some of the particles. When such animals are preserved over several months following the final Thorotrast injection thymocytes gradually reappear while the loaded macrophages remain as clumps of cells within the lobular cortices.

Red bone marrow It is invariably difficult to study the morphology of the red marrow sinusoids because of a profusion of associated developing blood cells. After even a single intravenous injection of Thorotrast however a few macrophages appear among the immature blood cells. In all instances they are free phagocytes none has been observed attached to the sinusoidal wall. As the number of Thorotrast injections is increased the storage properties of marrow are not markedly augmented possibly because of a concomitant stimulus toward hematopoiesis.

Lung The lungs of rats and mice began to collect Thorotrast particles soon after the initial intravenous injection. In all cases particles appear in the so called septal cells seen prominently in the interalveolar septae and later in great numbers within the alveolar lumen.

Pseudosinusoids During the course of the experiments just described careful study was made of blood channels in the anterior lobe of the hypophysis the parathyroid gland and in the islands of Langerhans. These channels are of inconstant diameter and are lined by a simple squamous epithelium whose cells are supported by a rich reticular fiber network. In no instance has it been possible to observe phagocytosis of Thorotrast particles in these lining cells. At the moment it seems unlikely that these cells never come in contact with the circulating colloid particles this leads to the conclusion that they

FIGURE 2 (*a*) Nodule in Glisson s capsule from the liver of a rat injected intraperitoneally with Thorotrast over a period of 6 months. The large heavily loaded macrophages near the center of this nodule appear to be coalescing while the smaller more peripherally located phagocytes remain discrete. ×456. (*b*) Subcutaneous nodule formed in a rat following a single injection of Thorotrast 11 months before sacrifice. Two weeks previously it was noted that the nodule had enlarged and softened. Microscopically numerous mitoses were found in the small cells with hyperchromatophilic nuclei that made up the bulk of the nodule. In the center of the figure a heavily loaded macrophage also in division is seen. ×456. (*c*) Nodule similar in origin and age to that seen in (*b*). This nodule however was firm in texture mitoses were absent and the predominant cells contained rather hypochromatophilic nuclei usually with prominent nucleoli. Due to the presence of many fibers the macrophages were typically flattened and elongate. ×456. (*d*) Liver from a rat repeatedly injected intravenously with Thorotrast for a period of 3 months. In the lower and central portion are seen distended macrophages still attached to the sinusoidal wall. By contrast a disintegrating group of macrophages is seen lying free in a sinusoidal lumen near the top. ×456. (*e*) Splenic red pulp from a mouse repeatedly injected intravenously with Thorotrast over a period of 1 month. Note the loaded free macrophages mixed with lymphocytes within the cords of Billroth. ×456. (*f*) Adrenal from a mouse injected repeatedly with Thorotrast for 2 months by the intravenous route. Macrophages have become freed from linings of the cortical sinusoid and are shown here collected in groups within sinusoids of the zona reticularis. ×456.

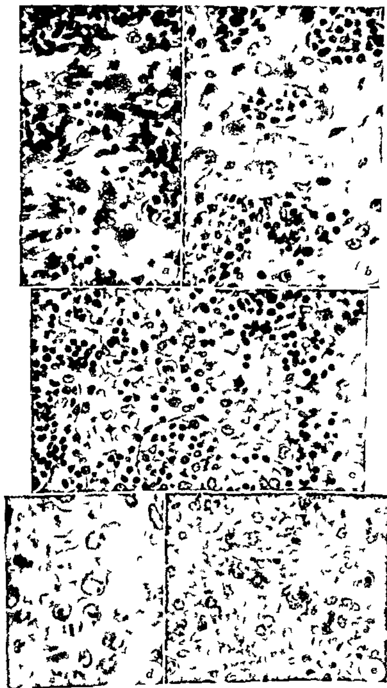


Figure 3

should not be considered as elements of the RES Such channels might thus be designated as pseudosinusoids

Corpus luteum Correlation of intravenous Thorotrast injection with phases of the rat and mouse estrous cycle has shown that corpus luteum sinusoids are lined by true phagocytes During growth and development of the corpus luteum there is little phagocytic activity but with the advent of its regressive phase they ingest and destroy the cell detritus Any Thorotrast available at this time is stored in the same manner as that seen in fixed phagocytes elsewhere [23]

RE exhaustion When repeated Thorotrast injections are made there is progressive mobilization of phagocyticopotential cells throughout the body It is difficult to trace the sources of these cells but some undoubtedly arise from histiocytes and reticular elements of the tissues themselves some also arise from cells brought in by migration from the vascular supply When the colloid is injected repeatedly by the intravenous route the reaction takes place on a system wide basis and eventually produces great numbers of activated macrophages These same circumstances eventually cause toxic changes as well as indicated by a slow but progressive loss of appetite weight and degree of physical activity This chronic state can persist for a protracted period if injections are eliminated Should the dosage be increased however the animals soon succumb [24]

The toxic animals characteristically show increased hematopoietic activity in the marrow and myeloid metaplasia in the spleen liver and lymph nodes It will be remembered that the rodent spleen normally carries an active myeloid component but under the present circumstances the organ becomes largely granulopoietic In toxic animals that succumb following increased dosage of the colloid there is a decrease in cellularity of the marrow and depletion of lymphocytes from lymphocytopoietic organs while free clumps of Thorotrast granules appear in various tissues Simultaneously there arise few newly activated macrophages that is those that contain small numbers of phagocytosed granules These findings are interpreted to indicate that one phase of the toxic reaction is expressed by depression in the RE cell replacement mechanism an evidence of RE exhaustion

RE Changes in Cancer Bearing Mice

The Ehrlich ascites carcinoma of mice differs in its growth characteristics depending on the mode of inoculation When an adequate number of living

FIGURE 3 ( ) Mesenteric lymph node from a mouse injected intraperitoneally with Thorotrast for 1 week Both free and fixed macrophages appear in all stages of loading within the cortical sinusoids ×456 (*b*) Mesenteric lymph nodes from a mouse injected intraperitoneally with a cell free ascitic fluid from a host to the Ehrlich ascites tumor Note the enlarged sinusoidal lining cells and the presence of endoplasmic granules No evidences of phagocytosis are visible ×456 ( ) Lymph node from a mouse treated in the same manner as that described in (*a*) Note the enlargement and vacuolation in RE cells lining the cortical sinusoids The cell in mitosis at the left of the figure is probably a lymphocyte ×456 (*d*) Lymph node from a mouse injected 3 times intraperitoneally with cell free ascitic fluid and sacrificed 1 week later Note the plasmacytoid changes in cells of a medullary cord Fixed reticular cells with pale nuclei appear near the top of the figure ×1 200 (*e*) Liver from a mouse treated in the same manner as described above This photomicrograph shows myeloid metaplasia as exhibited by a cluster of cells with ring or spiral nuclei at the top of the figure Near the lower right margin is a group of mononuclear hemocytoid forms ×456

cells is injected into the peritoneal cavity it develops in ascites form, that is as individual cells suspended in ascitic fluid[25] The semisolid form consisting of tumor cells enmeshed in a fibrous network, appears after prolonged incubation of the ascites tumor within a single host When injected subcutaneously or intramuscularly, the tumor develops in solid form

Reticuloendothelial changes in cancer bearing mice are most prominent in animals that have been inoculated intravenously During the first week after inoculation free tumor cells can be found circulating throughout a number of organs Later they collect in clusters that may or may not disintegrate Should a metastasis be formed the cells in such groups begin to show mitotic and migratory activity

Lung The earliest most numerous and most invasive tumors appear in the lungs of intravenously inoculated mice Free tumor cells are seen first within the pulmonary vessels they appear later in the adventitia of the air tube system and still later, in the interalveolar septae Particularly in the latter location they undergo rapid multiplication to produce nodules Early in this period of metastasis formation septal cells become numerous and prominent They enlarge and form vacuoles but are phagocytic toward cell detritus only Septal cells migrate actively and particularly in lungs with advanced tumor formation collect within the flattened alveolar spaces

Lymph nodes Lymph nodes undergo marked changes in histological structure while tumor cells or the ascitic fluid or both are traversing the sinusoids During early stages of this process fixed cortical sinusoidal lining cells enlarge through cytoplasmic vacuolation (FIGURE 3*b* and *c*) In addition to vacuoles the littoral cell cytoplasm may also develop endoplasmic granules or acidophilic rods the latter may be several microns in length With prolonged stimulation the medullary sinusoidal cells are similarly altered but to a smaller degree

Nodes transmitting tumor cells typically become lymphocyte poor possibly through depression of the regeneration mechanism or through increased loss by migration In some instances the lymphocytes of medullary cord and cortical tissue spaces undergo marked plasmacytoid changes (FIGURE 3*d*)

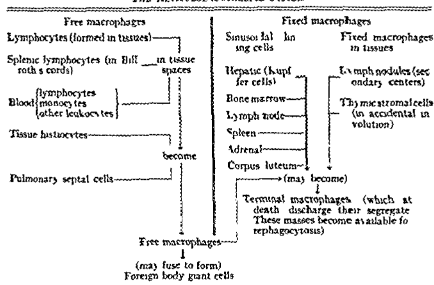
Liver The livers of mice injected intravenously with tumor cells or with cell free ascitic fluid show moderate RE reaction In the former instance metastases are rare despite the presence in sinusoids of many apparently viable tumor cells The Kupffer cells may under these conditions enlarge and become vacuolate Clusters of myeloid elements are found in the sinusoids and it is believed that these are formed by direct transformation of the sinusoidal lining elements The same changes appear after intravenous injection of ascitic fluid alone although the reaction is less striking and delayed in appearance (FIGURE 3*e*)

Spleen The spleens of mice after intravenous injection of cancer cells are typically small in size Study of the red pulp reveals great numbers of free tumor cells and immature myeloid elements intermixed with each other and with plasmacytoid cells that appear to be of lymphocytic origin The white pulp is inconspicuous having been reduced to a narrow sheath of lymphocytes about the blood vessels Malpighian enlargements are rarely seen The lining cells of the splenic sinusoids appear to be unaltered

Discussion

The significance of these observations is not entirely clear There are indications that stimulation of the RES to phagocytosis by injection of colloid produces a consistent histocytological response and that this response is one element of the defense mechanism Under the conditions of the experiment no cytoquantitation is possible but the method still serves as a means for identification of phagocytes and phagocyticopotential cells It should be adequate also to give us further insight into cytological changes associated with the collection and storage of a foreign particulate material

TABLE 1
THE RETICULOENDOTHELIAL SYSTEM



These studies present further evidence of RES reaction in cancer bearing hosts Details of the response pattern vary with the host and with the amount of tumor present but are consistent as to type and in many respects are comparable with those seen after injection of a foreign colloid Changes are seen in both free and fixed RE cells of the cancer bearing mice but the greatest single response is in the lymphocyte producing mechanism and behavior of its line

Great care must be observed in interpretation of the data presented when making generalizations on structure and function of the RE as a unit system One approach to this problem is presented in TABLE 1 which is intended to emphasize the interrelationships between free and fixed elements of the system As is the case with all schemata however it should serve principally as an aid in understanding of a broad picture The details within

this pattern of presentation are being continually altered or eliminated as additional information becomes available

Summary

An attempt has been made to delineate the RES by stimulating its cells to phagocytic activity using Thorotrast as a test material. The histocytological response pattern in various organs was followed in order to determine the mode of reaction and relationships between free and fixed RE cells.

Using the same basic plan of study, the various RE tissues were subjected to the stimulating effects of cells and/or ascitic fluid of the Ehrlich carcinoma. It was found that the histocytological response to this cancer was, in many respects, similar to that seen after Thorotrast stimulation.

These and other experimental data are presented in the form of a chart that attempts to correlate the reactive mechanisms in the RES as a functioning unit.

References

1 Aschoff L 1924 Reticulo-endothelial system Ergeb inn Med u Kinderheilk 26 1
2 Jaffe R H 1931 The reticulo endothelial system in immunity Physiol Revs 11 277-307
3 Baker R D 1934 The reticulo endothelial system a reconsideration Southern Med J 27 913-918
4 Baginski S 1938 Die Histo Physiologie des reticuloendothelialen Systems (RES) II Mitteilung Histologische Untersuchungen über die Blockade des reticuloendothelialen Systems Zellforsch u mikroskop Anat 28 387-407
5 Damade R 1938 Le Système reticulo endothélial Ses réactions physiologiques Nutrition 8 1-10
6 Doan C A 1940 The reticulo endothelial system its physiology and pathology J Lab Clin Med 26 89-101
7 Chevremont M 1948 Le Système histiocytaire ou réticuloendothélial Biol Rev 23 267-295
8 Tompkins E 1949 The reticulo endothelial system J Am Med Women s Assoc 4 447-463
9 Gabrieli E 1955 Reticulo endothelial activity during antibody response Ann N Y Acad Sci 59(3) 337-342
10 Baillif R N 1953 The reticulo-endothelial system a structural and functional analysis Bull Tulane Med Faculty 12 141-149
11 Tripoli C J & E von Haam 1931 Effects of toxic and non toxic doses of thorium dioxide in various animals Proc Soc Exptl Biol Med 29 1053-1056
12 Harris W H & A V Friedrichs 1932 Microscopic changes produced in tissues of animals injected with thorium dioxide for spleno hepatography Proc Soc Exptl Biol Med 29 1047-1049
13 Irwin D A 1932 The experimental intravenous administration of colloidal thorium dioxide Can Med Assoc J 27 130-135
14 Wen I C & T S Jung 1933 Changes in tissues after intravenous injection of colloidal thorium dioxide Proc Soc Exptl Biol Med 31 330 332
15 Shute E & M F Davis 1933 Histologic changes in rat [illegible] following the intravenous injection of thorium preparations Arch Path 15 27 34
16 Reeves R J & J F Morgan 1937 The retention of thorium [illegible] by the reticulo endothelial system Radiology 29 612 614
17 Gaunt W E & G P Wright [illegible] A comparison of the distribution between various organs of arsenicated serum protein and [illegible] (Thorotrast) following their intravenous injections J Infectious Dis [illegible] 67 217-221
18 Hale A R & R N Baillif 1953 The retention and elimination of colloidal thorium dioxide in the albino rat following prolonged administration Anat Record 117 163-174

19 BAILLIF R N 1955 Histologic changes associated with long term storage of Thorotrast in rats and mice KLS Bull 2 30-39
20 SAMPAIO M M 1956 The use of Thorotrast for the electron microscopic study of phagocytosis Anat Record 124 501 517
21 BAILLIF R N 1951 Lymph node changes following repeated injections of acid colloidal substances in the albino rat Am J Anat 88 109-162
22 BAILLIF R N 1949 Thymic involution and regression in the albino rat following injection of acid colloidal substances Am J Anat 84 457 510
23 BAILLIF R N 1948 Ovarian response in the albino rat to injected colloidal substances Am J Anat 83 109-142
24 BAILLIF R N 1953 Splenic reactions to colloidal thorium dioxide in the albino rat Am J Anat 92 55-116
25 SEEGER P G 1937 Untersuchungen am Tumoraszites des Maus Vitalfarbbarkeit der Asziteszellen Arch Exptl Zellforsch 20 280-335

INFLUENCE OF RETICULOENDOTHELIAL AND OTHER CELLS ON THE METABOLIC FATE OF STEROIDS*

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We have long been interested in studying the relationship that exists between the influence of steroid hormones on certain cellular reactions and in turn the influence of these reactions on the metabolism of the steroid hormone We feel that it is of major importance to ascertain whether there is a relationship between the action of a steroid hormone on the cell and the concomitant steroidal molecular alteration that ensues during the period of time that the hormone exerts its influence This interaction is also of importance with respect to the general body metabolism and excretion of steroid hormone since the level of the amount of hormone available to exert functions of various types is dependent upon not only the rate of secretion but the rate of removal

It has been ascertained that major sites of metabolism of certain adrenocortical steroids are the fibroblasts and the reticuloendothelial (RE) cells[1-3] We have known for some time that the hormone cell relationship between hydrocortisone (cortisol) and fibroblasts is of considerable significance in the expression of the antiphlogistic function of this hormone Numerous publications have dealt with this mechanism for inhibition of the inflammatory response[4-7] More recently studies have been performed on the reticuloendothelial cells to elucidate the role that they play in the catabolism and excretion of cortisol and corticosterone[8,9] This is of particular importance since it is known that the liver is a major site of cortisol and corticosterone catabolism and that it has a unique function not possessed by other organs or cells that is the formation of water soluble steroid glucuronide or sulfate conjugates[10,11] It is therefore of major importance to ascertain the roles played by reticuloendothelial cells of the liver and the hepatic cell Needless to say the role of reticuloendothelial cells in the metabolism of cortisol may be different in organs other than the liver but the extreme importance of the liver in the scheme of cortisol metabolism emphasizes the significance of the cell at this site

Another closely related cell that is derived from either fibroblasts or RE cells is the lymphocyte Cortisol inhibits the production of lymphocytes and brings about their maturation and destruction[12,13]

The relationships among the different actions of cortisol on a group of closely related mesenchymally derived cells are discussed below The function of cortisol and its relationship to metabolism by these various cell types are pointed out and discussed

General Metabolism of Steroids

Biological half life of steroids Our studies have shown that RE cells hepatocytes fibroblasts and lymphocytes are the major cell types that determine the

* The work reported in this paper was supported in part by Grant 34 5916 Cooperative Leukemia from the National Cancer Institute Public Health Service Bethesda Md

† Key Investigator of the American Cancer Society

biological half lives of steroids. These cells retain, metabolize and excrete the steroid hormones in different ways according to the number of carbon atoms, oxygen atoms and special configuration of the molecule. From TABLE 1 one may see that as the number of carbon and oxygen atoms decrease, the biological half life of steroids also decreases.

Cholesterol ($C_{27}O$) > cholic acid ($C_{24}O_5$) > cortisol ($C_{21}O_5$) > corticosterone ($C_{21}O_4$) > 11βOH androstenedione ($C_{19}O_3$) > testosterone ($C_{19}O_2$) > 21-deoxytetrahydrocortisone ($C_{21}O_4$). Note that 21 deoxytetrahydrocortisone has the shortest half life of the steroid listed in TABLE 1 since this compound is completely reduced in ring A (tetrahydro configuration) thus favoring its elimination as a water soluble compound, as discussed below.

The reticuloendothelial cells have the ability to retain some steroid molecules longer than others. Thus cholesterol is retained by the RES for a much longer period of time than cortisol.[1,4] Cortisol is retained for a very short time by the reticuloendothelial cell but apparently concentrates in fibroblasts[8] which,

TABLE 1
COMPARISON OF STEROID AND STEROL BIOLOGICAL HALF LIVES IN PLASMA

	Biological half life*	Biological half life reported in literature
Cholesterol ($C_{27}O$)	7 days	Cholesterol 11–13 days[4] Cholic acid ($C_{24}O_5$) 2–3 days
Cortisol ($C_{21}O_5$)	40–50 min	Cortisol 60–90 min[19,7]
Corticosterone ($C_{21}O_4$)	30–35 min	Corticosterone 60 min
11βOH Androstenedione ($C_{19}O_3$)	20–30 min	Progesterone ($C_{21}O_2$) 20 min[8]
21 Deoxytetrahydrocortisone ($C_{21}O_4$)	10 min	Testosterone ($C_{19}O_2$) 11 min[20]

* Values obtained in our laboratory.

in turn, have the ability to metabolize this steroid at a rapid rate.[21] The half lives given in the left column of TABLE 1 are of the $4C^{14}$ labeled steroids themselves, that is, of the actual steroid isolated and identified by paper chromatography and not that of total radioactivity. The figures on the right side of TABLE 1 are those given by other investigators and not all of them represent the half life of the free steroid injected but include the bulk of its metabolites as well. However, both types of data reveal that the same trend is closely followed. Another factor of possible importance that can influence the half life of a steroid is that steroid may be bound differentially to plasma proteins.[22,23] The nature of this binding may favor or act against the ability of cells to metabolize steroids. Therefore we can say that the biological half life of a steroid can be influenced by four factors: (1) the molecular weight of the steroid and its configuration, (2) whether or not a neutral steroid hormone is reduced in ring A, (3) the specific uptake of the cell type toward a steroidlike cholesterol (RES) or cortisol (fibroblast) and (4) the binding affinity of a steroid to plasma protein and this relationship in turn to the steroid metabolizing cell.

The liver plays several roles in steroid and sterol metabolism. In recent

years the availability of radioactive compounds has allowed a very detailed analysis of hepatic function by administering C^{14} labeled substances to hepatectomized and normal rats. The steroids studied in hepatectomized preparations were cortisol,[10] corticosterone,[4] progesterone[25] and 21 deoxytetrahydrocortisone.[24] It has been found that these animals are not able to conjugate these steroids to water soluble compounds. Since it has been shown that this is a fast reaction that occurs in normal animals, the liver seems to be the principal if not the only organ that can conjugate steroids. However, the

Cortisol → Dihydrocortisol → Tetrahydrocortisol → Tetrahydrocortisol Glucuronide

FIGURE 1. Liver metabolism: scheme of typical ring A reduction and conjugation of corticosteroids by the liver.

extrahepatic tissues were able to metabolize the steroids tested; that is, they oxidize and reduce the substituted groups of the molecule, but they cannot reduce actively the $\alpha\beta$ unsaturated ketone configuration in ring A and form tetrahydro compounds.[10, 4, 5] Extrahepatic tissues can produce a minor but measurable amount of double bond reduction (dihydro form), but no conjugation was observed. The liver also performs transformations similar to those accomplished by extrahepatic cells.[6, 7] The unique functions of the liver in cortisol metabolism are shown in FIGURE 1. The first reaction (cortisol to dihydrocortisol) has been shown to be irreversible.[7, 9] However, the reaction from dihydrocortisol to tetrahydrocortisol has been reported to be reversible in various tissues.[11] Tetrahydrocortisol, however, is conjugated as soon as it is

formed; thus the second reaction becomes for practical purposes essentially an irreversible reaction since the conjugate is then excreted rapidly. FIGURE 2 illustrates the over-all metabolism of cortisol divided into hepatic and extrahepatic segments. Inside the quadrangle are all the steroids that have not been reduced in ring A and outside of it are those that have been reduced in ring A through the dihydro form. The tetrahydro derivatives have been isolated mainly as glucuronides. All of the steroids in FIGURE 2 have been isolated from studies on human[30] and mice.[31] The full line arrows indicate the steps that have been tested and that occur *in vivo* or *in vitro*; the interrupted arrows indicate steps that have not yet been proved in our laboratory, but that probably exist.

It is well known that cortisol and cortisone are interconvertible and that 20-*epi* substance E of Reichstein is also interconvertible with 20-*epi* substance U of Reichstein. The same situation exists for the 20β homologues. The possible oxidation of the 20α and/or β-ol configuration to the 20 ketone has not been tested as yet. However, in previous experiments it was shown that possibly a hydroxy group in the 20 position may be transformed to a ketone in the adrenal gland.[27] This reaction was the transformation of 4-pregnene-20β-ol-3-one to progesterone and subsequently to cortisol in the bovine adrenal gland *in vitro*.[32] This reaction has also now been shown to occur in ovarian tissue from 4-pregnene-20α-ol-3-one to progesterone.[33] The possibility therefore exists that other compounds reduced in the 20 position can be oxidized. We should like to postulate the existence of an extrahepatic cyclic transformation of cortisol as represented in FIGURE 2. The possible physiological significance of this cycle is considered below.

Extrahepatic Metabolism

The fibroblasts. The predominant cell found in connective tissue is the fibroblast.[7] This cell of mesenchymal origin and widely distributed throughout the organism has been shown to be a target cell for corticosteroids.[4, 6, 7] The enzymic capabilities and functions of these cells are numerous;[34] they include the formation of collagen, the formation of ground substance, micellophagosis,[35] and the synthesis of cholesterol,[36, 37] and they have shown catalase, prolinase, phenolsulfatase, cytochrome *c* reductase, lactic dehydrogenase, malic dehydrogenase, glutamic oxalacetic transaminase, peroxidase, alkaline phosphatase, β-glucuronidase, proteolytic, prolidase, and acid phosphatase activities.[34]

We first discuss the ability of fibroblasts to metabolize steroids and to synthesize sterol. For many years the major interest of this laboratory has been the relationship that exists between corticosteroids and their anti-inflammatory effect.[4, 6] It has been well established that cortisol is the most highly potent anti-inflammatory agent[1] normally produced by the adrenal gland and that it probably exerts its antiphlogistic effect within an inflamed volume of tissue by inhibiting the focal damage of fibroblasts and endothelial cells, thereby preventing this influx of nonautochthonous cells into the injured tissue.[7] It has been observed that after the administration of a mild phlogogenic stimulus there is a destruction of fibroblasts and that a variable number of polymor

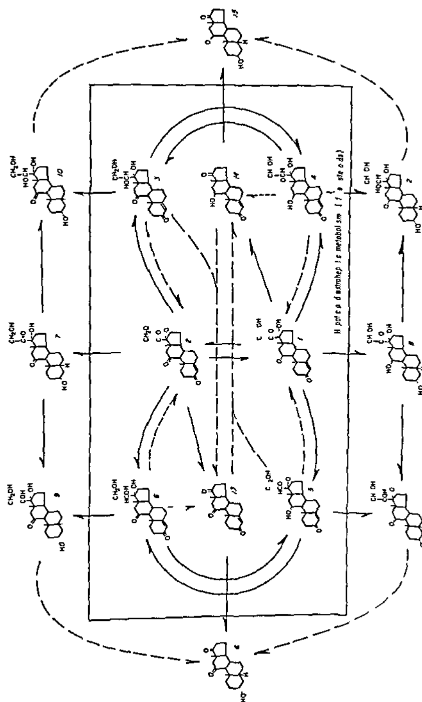


FIGURE 2

phonuclears and macrophages enters the inflamed area However when cortisol is administered locally or systemically the fibroblastic destruction is decreased and the entrance of polymorphonuclears into the area is also decreased or abolished [5] There is a particular cellular change that occurs in the fibroblast after treatment with cortisol in anti inflammatory doses the hormone induces a rounding up or epitheloid type change in many but not all fibroblasts [6] This has been shown to occur in connective tissue or in tissue culture fibroblasts [37] These results have led to the theory of Eyring and Dougherty [38] by which they explain that inflammation exerts an autocatalytic cell destruction in which the breakdown of one cell promotes the breakdown of another by means of the destructive products liberated by the broken cell This destructive inflammatory chain reaction could be interrupted because some fibroblasts are resistant to the accumulation of substances that bring about their swelling and lysis Cortisol which rounds up fibroblasts and concentrates in them [6] can stop the catalytic process of cell destruction by making these cells resistant to the destructive effect of the accumulation of substances thereby stopping the inflammatory reaction

Metabolism of steroids by fibroblasts The picture of fibroblastic metabolism toward steroids has been studied intensively in this laboratory especially with cortisol [1,39] and corticosterone [40] It has been shown that in connective tissue or in tissue culture this cell has a great ability to oxidize and reduce the various substituted groups of the gonane (cyclopentane-perhydrophenanthrene) nucleus of these steroids The products of conversion from incubations of loose connective tissue and tissue culture fibroblasts with various steroids are shown in TABLE 2 All of these studies were done using radioactive micromethods developed in this laboratory [41-43] A great variety of products is formed from these incubations From incubation studies with cortisol it was observed that loose connective tissue fibroblasts from mice [1] preferentially form the α isomer at the 20 position while fibroblasts in tissue culture usually produce metabolites of the β configuration in the 20 position (TABLE 2) This probably represents more a quantitative difference than a qualitative one Both 20α and 20β isomers were formed from the incubations of progesterone in tissue culture although the 20α configuration was preferentially formed [39] There seemed to be an apparent inability of tissue culture fibroblasts to oxidize the

FIGURE 2 Hepatic and extrahepatic metabolism of cortisol (1) 4-pregnene 11β 17α 21 triol-3 20-dione (cortisol) (2) 4 pregnene 17α 21 diol 3 11 20-dione (cortisone) (3) 4-pregnene 17α 20β 21 triol 3 11-dione (substance U of Reichstein) (4) 4 pregnene 11β 17α 20β 21 tetrol 3 one (substance E of Reichstein) (5) 4 pregnene 11β 17α 20α 21 tetrol 3-one (20-epi substance E of Reichstein) (6) 4-pregnene 17α 20α 21 triol 3 11-dione (20-epi substance U of Reichstein) (7) pregnane 3α 17α 21 triol 11 20-dione (tetrahydrocortisone) (8) pregnane 3α 11β 17α 21 tetrol 20-one (tetrahydrocortisol) (9) pregnane 3α 17α 20α 21 tetrol 11-one (α-cortolone) (10) pregnane 3α 17α 20β 21 tetrol 11-one (β-cortolone) (11) pregnane 3α 11β 17α 20α 21 pentol (α-cortol) (12) pregnane 3α 11β 17α 20β 21 pentol (β-cortol) (13) 4 androstene 3 11 17 trione (adrenosterone) (14) 4 androsten 11β-ol 3 17 dione (11β-OH androstenedione) (15) etiocholane 3α 11β-diol 17-one (11β-OH-etiocholanolone) and (16) etiocholane 3α ol 11 17-dione (11 keto-etiocholanolone)

All of the compounds listed here are the major ones found 6β hydroxylated steroids and others not fully identified are not represented The configuration 5α or allo is also present in the metabolism of cortisol [33] The dark lines represent pathways that we and/or other investigators have found to exist Those indicated by the dotted lines have not been tested as yet in our laboratory

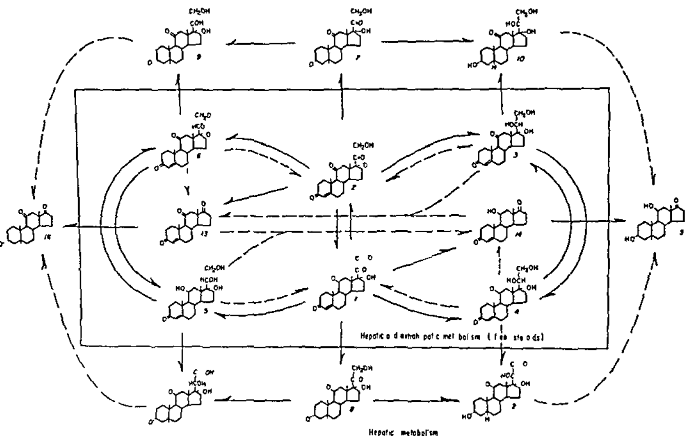


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FIGURE 2. Hepatic and extrahepatic metabolism of cortisol. (1) 4-pregnene-11β,17α,21-triol-3,20-dione (cortisol); (2) 4-pregnene-17α,21-diol-3,11,20-trione (cortisone); (3) 4-pregnene-17α,20β,21-triol-3,11-dione (substance U of Reichstein); (4) 4-pregnene-11β,17α,20β,21-tetrol-3-one (substance F of Reichstein); (5) 4-pregnene-11β,17α,20α,21-tetrol-3-one (20-epi substance E of Reichstein); (6) 4-pregnene-17α,20α,21-triol-3,11-dione (20-epi substance U of Reichstein); (7) pregnane-3α,17α,21-triol-11,20-dione (tetrahydrocortisone); (8) pregnane-3α,11β,17α,21-tetrol-20-one (tetrahydrocortisol); (9) pregnane-3α,17α,20α,21-tetrol-11-one (α-cortolone); (10) pregnane-3α,17α,20β,21-tetrol-11-one (β-cortolone); (11) pregnane-3α,11β,17α,20α,21-pentol (α-cortol); (12) pregnane-3α,11β,17α,20β,21-pentol (β-cortol); (13) 4-androstene-3,11,17-trione (adrenosterone); (14) 4-androstene-11β-ol-3,17-dione (11β-OH-androstenedione); (15) etiocholane-3α,11β-diol-17-one (11β-OH-etiocholanolone); and (16) etiocholane-3α-ol-11,17-dione (11-keto-etiocholanolone).

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11 position of cortisol to cortisone[32] However this was also a quantitative rather than a qualitative difference as small amounts have been isolated The fibroblast is without doubt one of the most active cells in the extrahepatic metabolism of steroids This cell is capable of forming the metabolites in ide the quadrangle depicted in FIGURE 2

The reversibility between 20-hydroxy and 20-ketonic steroids has been established[37, 38] The effectiveness on gluconeogenesis and lymphocytokaryorrhexis of C 20-reduced steroid (for example Reichstein s substances E and U 20α or 20β) is much less than that of cortisol and corti one[41, 43] The ratio of the

TABLE 2
METABOLISM OF STEROIDS BY FIBROBLASTS

Ste l incubat d	St r d solated
Corticosterone	4 Pregnene 11β 20β 21 triol 3-one
17OH Progesterone	4-Pregnene 17α 20ξ diol 3 one†‡
Progesterone	4-Pregnene 20β ol 3-one 4-Pregnene 20α ol 3 ne Allopregnane 3 20 li ne Allopregnane 20α-ol 3-one
4 Pregnene 17α 21 diol 3 20-dione	‡4 Pregnene 17α 20ξ 21 triol 3 20-dione†
Cortisol	4 Pregnene 11β 17α 20α 21 tetrol 3 one (20-epi substance F of Reichstein)† 4 Pregnene 11β 17α 20β 21 tetrol 3 one (substance E of Reichstein) Pregnane 11β 17α 21 triol 3 20-dione (dihydrocortisol)† 4 Pregnene 17α 20α 21 triol 3 11 dione (20-epi substance U of Reichstein)† 4 Pregnene 17α 20β 21 triol 3 11 dione (substance U of Reichstein) 4 Pregnene 17α 21 diol 3 11 20 trione (cortisone)† 4 Androstene 11β ol 3 17 dione †

These metabolites are compounds isolated and characterized from i cubations of 4 C 4 steroids with tissue cultures of fibroblasts or loose connective tis ue
Tissue culture fibroblasts
† Loose connective tissue of mice
‡ Configuration at C 20 not determined at this time

concentrations of the active to the inactive forms of a teroid can be controlled by the cell

Another situation exists in the connective ti sue in which there could be an active inactive ratio between 2 steroids that is between corti ol and cortisone The reaction of corti ol (active) to cortisone (inactive) i known to be reversible[11] Cortisol is at least 7 or 8 times more potent in it antiphlogistic effect than cortisone[7] it is very possible that only cortisol is the anti inflammatory steroid[1] and that cortisone must be converted to corti ol for this hormone to be antiphlogistic In other experiments[46] it was shown that 2 methyl cortisone cannot be actively converted to 2 methyl cortisol metabolically and when 2 methyl cortisone was tested for biological activity the compound was

far less active than cortisone or cortisol However 2 methyl cortisol is more active than cortisol as tested for gluconeogenic or anti inflammatory activity [47] This also suggests that cortisone must be converted to cortisol for this steroid to be active Therefore at the cellular level in the fibroblast there could be a ratio between the active and the inactive steroid (11β-ol ⇌ 11 keto) It has been shown that steroids can participate in the reversible reduction and oxidation of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) as a mechanism for hydrogen transfer [48] It is also known that these nucleotides can increase the reduction or oxidation of the substituted groups of the steroid nucleus [11] Therefore the ratio between oxidized and reduced coenzymes available could influence the cyclic conversion of cortisol and maintain an intracellular ratio between an active and an inactive steroid

Reticuloendothelial cells It has been shown that cholesterol can be synthesized from acetate by fibroblasts in tissue culture[46] and by slices of a sponge connective tissue-cell preparation [51]

Factors affecting intracellular and extracellular cholesterol levels are absorption synthesis and excretion It has been shown that obstruction of the bile duct produces hypercholesterolemia but that this does not occur when the liver is removed The removal of various other organs prior to obstruction of the bile duct was without effect [49] It has therefore been postulated by Byers[50] that the liver is the principal if not the sole source of plasma cholesterol and that this is a soluble cholesterol that is taken up chiefly by the parenchymal cells of the liver Two types of cholesterol exist one is synthesized and sent to the blood in soluble form by the liver and is not phagocytized the second which is of oral origin is phagocytized by Kupffer's and other RES cells [1,3,5] An interference with this last function (that is the phagocytosis of cholesterol) has been described by Niven *et al* [52] These investigators injected particulate matter that was taken up preferentially by Kupffer cells thus producing hyperlipemia and hypercholesterolemia by displacement of cholesterol to the blood This is interesting since it shows that the reticuloendothelial cell function is of great importance in maintaining these hyperlipemic and hypercholesterolemic states

The relative specific activities expressed as counts per minute per milligram of dry tissue of various organs of mice after these animals were given a single dose of cholesterol 4 C^{14} intragastrically are shown in TABLE 3 These animals were sacrificed 9 days later and their tissues were homogenized and counted for amounts of radioactivity It may be seen that the relative specific activity in lung is significantly higher than in the liver Additionally the small intestines and the adrenals are high in relation to liver Another group given 4C^{14} cholesterol in the same manner was treated with L triiodothyronine during the 9 day period The specific activities in all the same organs were decreased significantly as compared to animals fed the same amount of cholesterol but given saline that is the elimination of the cholesterol from these organs was increased by the administration of L triiodothyronine or D-triiodothyronine (TABLE 3)

Elsewhere we have shown that ACTH has an opposite effect that is it retains cholesterol for longer periods of time than in non ACTH treated animals [1,3] The deposition of cholesterol in lung is in the reticuloendothelial

TABLE 3
DISTRIBUTION OF RADIOACTIVITY IN TISSUES AFTER CHOLESTEROL 4C[1] INGESTION IN MICE

T	Blood	L	Lymph 1 s	L g	A ta	Hea t	Adrenal	K d y	Small i t	Spleen
C t l										
No of determinations	10	10	5	10	5	10	5	10	10	10
Mean†	4 53	18 05	12 50	29 60	10 30	6 62	28 40	20 86	34 04	15 01
Standa d error	0 62	2 38	2 09	2 3?	1 59	1 01	9 58	1 10	5 52	1 65
L Triiodothyronine‡										
No of determinations	10	10	5	10	5	10	5	10	10	10
Mean†	1 11	3 27	4 44	10 91	1 18	2 94	4 26	11 99	9 67	6 44
Standard error	0 13	0 21	0 29	0 53	0 41	0 45	0 44	0 31	0 86	0 39
Value of P in relation to controls	< 01	< 01	< 01	< 01	< 01	< 01	0? < P < 05	< 01	< 01	< 01
D Tri dothyronine†										
No of determinations	12	12	6	12	6	12	6	12	12	12
Mean†	1 ?0	6 01	6 53	11 89	3 92	1 99	9 82	12 43	17 27	5 04
Standar l error	0 40	0 68	0 ?1	1 06	1 38	0 34	1 99	0 95	2 34	0 47
Value of P in relati n to on trol	< 01	1 < P < 2	01 < I < 0?	< 01	01 < P < 0?	< 01	05 < P < 1	< 01	< 01	< 01

Amount of cholesterol 4C[1] administered 4 μg = 156 000 cpm
† Expressed as cpm/mg dry tissue
‡ Dose administered 0 25 μM/mouse

cell as shown by radioautographic techniques[22] We thus have a contrast between 2 cells one of which (the fibroblast) can synthesize this sterol very actively and the other, the reticuloendothelial cell that retains it The half life of this sterol normally is quite long in comparison to other steroids (TABLE 1) When a compound such as L or D-triiodothyronine is given however these reticuloendothelial cells have the ability actively to remove cholesterol and diminish its biological half life This raises the possibility that the Kupffer's cell is responsible for the formation of bile acid from cholesterol (hydroxylation of the molecule and splitting of the side chain) as this is the principal metabolic pathway for this sterol[35]

The lymphocytes Some details of the metabolic activities of lymphocytes in relation to steroids are presented by us elsewhere in this monograph It is important to mention here that there is a quantitative difference between the ways mature and immature lymphocytes metabolize cortisol The lymphocyte has the ability to oxidize the 11 position of cortisol to form cortisone, also it can reduce cortisone to form cortisol This is a reversible reaction by which another cell (the lymphocyte) can activate or inactivate steroid molecules since cortisol is lymphocytokaryorrhectic and since cortisone possesses no such activity The activity of cortisone is dependent upon its being converted to cortisol Thus the capacities of lymphocytes to perform oxidation and reduction of this steroid regulate also the degree of its resistance or susceptibility to be matured and destroyed

Hepatic Metabolism

Many studies on the ability of the liver as a whole to metabolize steroids have been reported However no attention has been given to the liver cellular components in charge of these metabolic functions This portion deals with the cytological components of the liver that are involved in oxidations and reductions of substituted groups of steroid and sterol molecules and of their conjugation to form water soluble derivatives[43]

The cell population and the percentage distribution of these cells in liver tissue of mice or rats[53] are reported to be as follows parenchymal cells or hepatocytes 60 6 per cent littoral cells or RES cell 33 4 per cent bile duct cells 2 per cent connective tissue cells 2 2 per cent and blood vessel wall cell 1 8 per cent These data indicate that about 40 per cent of the cells are nonparenchymal the majority are both hepatocytes and reticuloendothelial cell A modification of the technique described by Rous and Beard[44] has been utilized in our laboratory for the purpose of separating iron containing Kupffer's cells from parenchymal cells in the liver with a magnet the device by which these cells are separated is shown in FIGURE 3 In order to ascertain whether the enzymic capacities to conjugate steroid by the liver are altered by the iron treatment an experiment was performed in which the conjugation of corticosterone-4C[14] and pregnane 3α-ol 11 20-dione 4C[14] was tested by incubating these steroid with livers of iron treated animal and those of nontreated animals As shown in TABLE 4 the presence of iron did not alter the capacity of the liver to conjugate either of the 2 steroid incubated that is it did not alter the capacity of the cells to reduce ring A and to form water-soluble com

pounds. TABLE 5 represents the incubation of corticosterone-4C[14] and pregnane-3α-ol,11,20-dione-4C[14] with both reticuloendothelial and parenchymal cells. The hepatocyte appears to be the only cell fraction that conjugates the

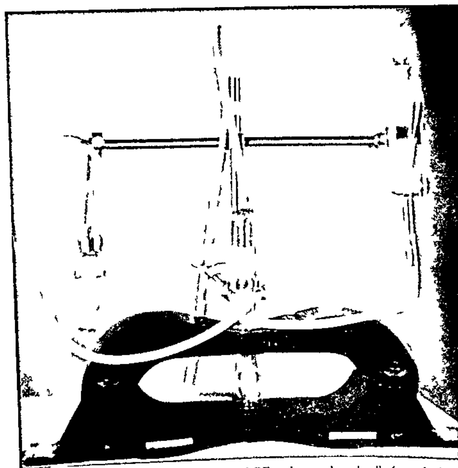


FIGURE 3. Procedure for the separation of RE and parenchymal cells from the liver. Liver breis of animals previously injected with carbonyl iron I.V. are separated by passing them through the coil while it is suspended between the poles of the magnet. The breis are prepared in K.I.O. buffer pH 7.4. The iron-containing cells (RE) adhere to the surface of the coil. Several washings with buffer solution allow a higher degree of separation.

steroids in significant quantities. It is of interest that both the reticuloendothelial and parenchymal cells were able to oxidize and reduce the substituted groups of the gonane nucleus (including ring A) as determined by paper chromatography. Although a small contamination of both cell fractions existed, the separation was adequate to determine that the hepatocyte is the cell that produces most of the steroid water-soluble conjugates.

Influence of Hormones

ACTH ACTH infusion and surgical stress have been shown to raise the levels of 17 hydroxycorticosteroids in peripheral blood It is now becoming evident that the mechanism of ACTH action may not be a simple and un

TABLE 4
EFFECT OF CARBONYL IRON TREATMENT ON STEROID CONJUGATION BY THE LIVER

Treatment	Steroid	No. of incubations (1 gm min [illegible] [illegible])	Water-soluble C14 (m[illegible])
None	Corticosterone 4C14	4	12 28 ± 1 79
Fe 5 mg IV	Corticosterone-4C14	4	11 19 ± 1 19
None	Compound N-4C14	4	16 75 ± 1 86
Fe 5 mg IV	Compound N-4C14	4	19 23 ± 4 85
Zero control	Corticosterone-4C14 Compound N-4C14	2 2	0 0

The effect of our standard dose of carbonyl iron was compared with nontreated animal livers One gm of liver mince was incubated with 50 mμmole of steroid in K PO buffer (pH 7 4) at 37 C for 3 hours in each case Zero controls were not incubated
Pregnane-3α-ol 11 20-d one-4C14

TABLE 5
CONJUGATION OF STEROIDS BY LIVER CELL FRACTIONS

Cell type	Steroid	Equivalent gm liver incubated	Per cent conjugated
RE	Corticosterone-4C14 Compound N-4C14	1 5 1 5	0 39 0 86
Parenchymal	Corticosterone-4C14 Compound N-4C14	1 5 1 5	4 93 10 9
Control	Corticosterone-4C14 Compound N-4C14	— —	0 0

The ability of whole cells of liver to conjugate 50 mμM of incubated steroid is compared here Each incubation flask contained 1 μM UDPGA 10 μM DPNH 10 μM isocitrate and K PO buffer (pH 7 4) Incubations were performed for a period of 3 hours at 37 C
Pregnane 3α ol 11 20-dione-4C14

complicated direct stimulation of the adrenal cortex but may also involve an influence of some factor or factors controlling the rate at which corticosteroids and their metabolites are removed from blood and tissues

It has been demonstrated by Engel[5] that extra adrenal effects of ACTH exist in relation to various enzymic mechanisms In addition it has been demonstrated in our laboratory that ACTH inhibits conjugation of cortisol

and corticosterone by the liver[8] This effect of ACTH is produced in adrenalectomized animal FIGURE 4 shows data of an experiment in which adrenalectomized mice were given ACTH and compared to nontreated control Cortisol 4C^{14} was given intravenously to both groups The livers of the animal were taken out at different times (3 7, 15 50 and 100 min) after cortisol infusion, and the various tissues were extracted for the separation of free and water soluble compounds It was found (FIGURE 4) that the ACTH

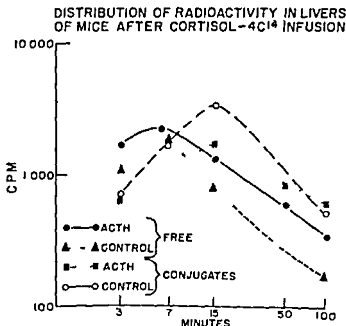


FIGURE 4 Isolation of free and water soluble conjugates from livers of adrenalectomized mice at different time intervals The formation of conjugated steroids is slower in the animals treated with ACTH

TABLE 6

MINCED LIVER TISSUE SAMPLES OF LT 3 AND DT 3 TREATED ANIMALS AND CONTROLS INCUBATED WITH 35 0 $m\mu M$ OF STEROID DATA FOR PER CENT CONJUGATION AND PER CENT OF STEROID LIBERATED BY β GLUCURONIDASE

Treatment	Number of [illegible] (1 gm tissue)	Steroid	Per cent conjugated	Per cent water soluble (glucuronides)
L Triiodothyronine 0 25 μmole/day for 4 days	4	Corticosterone 4C^{14}	11 9	66 7
	4	Compound X 4C^{14}*	1 1	66 8
D Triiodothyronine 0 25 μmole/day for 4 days	4	Corticosterone-4C^{14}	11 1	54 8
	4	Compound X-4C^{14}*	20 8	54 1
Saline	4	Corticosterone-4C^{14}	6 5	48 3
	4	Compound X-4C^{14}*	17 4	55 1

* Pregnane 3α ol 11 20-dione-4C^{14}

treated animal had a decreased ability to conjugate cortisol This effect also occurs when a tetrahydro compound is tested which suggests that ACTH is directly affecting the parenchymal cell of the liver

Thyroid hormones Livers of mice treated with L triiodothyronine (LT 3) or D-triiodothyronine (DT 3) were examined with respect to their ability to conjugate corticosterone and pregnane 3α-ol 11 20-dione (TABLE 6) It was observed that LT 3 and DT 3 increase the conjugation of corticosterone but not the conjugation of pregnane 3α-ol 11 20-dione The fact that corticosterone must be reduced in ring A before conjugation implies that the increased conjugation was a reflection of an increased reduction of ring A and not on the conjugation mechanism itself as there was no increase in conjugation with pregnane 3α-ol 11 20-dione (a ring A reduced steroid) These data agree with findings of previous investigators [25, 26] who have shown that the livers of rats treated with thyroxin can reduce the ring A of cortisone faster than nontreated animals can

Acknowledgment

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References

1 DOUGHERTY T F & D L BERLINER 1959 A comparison of the participations of fibroblasts and reticuloendothelial cell in the synthesis and metabolism of cortisol and cholesterol *In* Hormones and Atherosclerosis 103 G Pincus Ed Academic Press New York N Y
2 DOUGHERTY T F & D L BERLINER 1959 Some ways by which ACTH and cortisol influence functions of connective tissue *In* Connective Tissue Thrombosis and Atherosclerosis 143 166 I Page Ed Academic Press New York N Y
3 BERLINER D L & T F DOUGHERTY R E system and metabolism of cortisol and cholesterol Proc 3 d Intern Symposium on Reticuloendothelial System In press
4 DOUGHERTY T F 1953 Some observations on mechanisms of corticosteroid action in inflammation and immunologic processes Ann N Y Acad Sci 56(4) 748
5 DOUGHERTY T F 1954 The mechanism of action of adrenocortical hormones in allergy *In* Progress in Allergy 4 319 S Karger Ed Basel and New York
6 DOUGHERTY T F R BIGLER F I SCHNEEBELI & H A SALHANICK 1956 On the localization of steroid hormones in connective tissue Ann N Y Acad Sci 64(4) 466–475
7 DOUGHERTY T F 1950 Relation of adrenocortical hormones to the hypersensitive state *In* Adrenal Cortex 88–114 Josiah Macy Jr Foundation New York N Y
8 BERLINER D L & T F DOUGHERTY 1960 Hormonal effect on steroid conjugation Federation Proc 19 169
9 NABORS C J JR D L BERLINER & T F DOUGHERTY 1960 Liver cell and RES metabolism of steroids Intern Anatomy Meetings 136 249
10 BERLINER D L B I GROSSER & T F DOUGHERTY 1958 The metabolism of cortisol in eviscerated rats Arch Biochem & Biophys 77 81
11 SAMUELS L T Metabolism of steroids *In* Chemical Pathways of Metabolism D M Greenberg Ed Academic Press New York N Y In press
12 DOUGHERTY T F 1959 Adrenal cortical control of lymphatic tissue mass *In* The Kinetics of Cellular Proliferation 264 277 F Stohlman Ed New York N Y & London England
13 DOUGHERTY T F Lymphocytokaryorrhectic effect of adrenocortical steroids *In* The Lymphocyte and Lymphocytic Tissue Hoeber New York N Y In press
14 KURLAND G S J L LUCAS & A S FREEDBURG 1954 The metabolism of intravenously infused C labelled cholesterol in man J Clin Invest 33 950
15 BERGSTRÖM S 1958 Bile acid formation and secretion *In* Liver Function 310–324 R W Brauer Ed Am Inst Biol Sci Washington D C

16 JEFFERSON R F & J B WYNGAARDEN 1956 The miscible pool and turnover rate of hydrocortisone in man J Clin Invest 35 552
17 MIGEON C J A A SANDBERG H A DECKER D F SMITH A C PAUL & L T SAMUELS 1956 Metabolism of 4C[14] cortisol in man body distribution and rates of conjugation J Clin Endocrinol and Metabolism 16 1137
18 AYRES P J O GARROD S A S TAIT J F TAIT G WALKER & W H PEARLMAN 1957 The use of [16 ³H] aldosterone in studies on human peripheral blood Ciba Foundation Coll Endocrinology 11 309
19 SANDBERG A A & W R SLAUNWHITE JR 1958 The metabolic fate of C[14] progesterone in human subjects J Clin Endocrinol and Metabolism 18 253
20 SANDBERG A A & W R SLAUNWHITE JR 1956 Metabolism of 4C[14] testosterone in human subjects I Distribution in bile blood feces and urine J Clin Invest 35 1331
21 BERLINER D L & T F DOUGHERTY 1958 Metabolism of cortisol by loose connective tissue *in vitro* Proc Soc Exptl Biol Med 98 3
22 SANDBERG A A W R SLAUNWHITE JR & H N ANTONIADES 1957 The binding of steroids and steroid conjugates to human plasma proteins Recent Progr in Hormone Research 13 209
23 DAUGHADAY W H 1958 Binding of corticosteroids of plasma proteins III The binding of corticosteroid and related hormones by human plasma and plasma protein fractions as measured by equilibrium dialysis J Clin Invest 37 511
24 GROSSER B I D L BERLINER & T F DOUGHERTY 1959 The extrahepatic metabolism of corticosterone 4C[14] and pregnane 3α 11β-diol 11 20-dione 4C[14] Federation Proc 18 61
25 BERLINER D L & W G WIEST 1956 Extrahepatic metabolism of progesterone J Biol Chem 221 449
26 CASPI F Y H LEVY & O M HECHTER 1953 Cortisone metabolism in liver II Isolation of certain cortisone metabolites Arch Biochem Biophys 45 169
27 AXELROD L R & L L MILLER 1955 The metabolism of hydrocortisone in the isolated perfused dog liver Arch Biochem Biophys 60 373
28 TOMKINS G M 1959 Enzymatic metabolism of corticosteroids Ann N Y Acad Sci 82(4) 836-845
29 ROMANOFF L D J SEELYE R RODRIGUEZ & G PINCUS 1957 The regular occurrence of 3α allotetrahydrocortisol (3α 11β 17α 21 tetrahydroxy allopregnane 20-one) in human urine J Clin Endocrinol and Metabolism 17 434
30 BERLINER D L & M L BERLINER 1957 Metabolism of cortisol-4C in humans Federation Proc 16 153
31 DOUGHERTY T F H F BROWN & D L BERLINER 1958 Metabolism of hydrocortisone during inflammation Endocrinology 62 455
32 NABORS C J JR & D L BERLINER 1957 A C 20 reductase in the pancreas and a C 20 dehydrogenase in the adrenal Arch Biochem Biophys 70 298
33 WIEST W G 1959 Conversion of progesterone to 4-pregnene 20α-ol 3-one by rat ovarian tissue *in vitro* J Biol Chem 234 3115–3121
34 BOUCEK J R N L NOBLE & J F WOESSNER JR 1959 Properties of fibroblasts *In* Connective Tissue Thrombosis & Atherosclerosis 193–211 I Page Ed Academic Press New York N Y
35 HIGGINBOTHAM R D & T F DOUGHERTY 1957 Mucellophagosis RES 3 19 20
36 BERLINER D L H E SWIM & T F DOUGHERTY 1958 Synthesis of cholesterol by a strain of human uterine fibroblasts propagated *in vitro* Proc Soc Exptl Biol Med 99 51–53
37 HOLDEN M & L B ADAMS 1957 Inhibitory effects of cortisone and hydrocortisone on growth of fibroblasts Proc Soc Exptl Biol Med 95 36
38 EYRING H & T F DOUGHERTY 1955 Molecular mechanisms in inflammation and stress Am Scientist 43 457
39 SWEAT M L B I GROSSER D L BERLINER H E SWIM C J NABORS JR & T F DOUGHERTY 1958 Metabolism of cortisol and progesterone by cultured uterine fibroblasts strain U 12 705 Biochim et Biophys Acta 28 591
40 BERLINER D L H L SWIM & T F DOUGHERTY 1960 Metabolism of corticosterone 4C[14] by fibroblasts strain U 12 79 Biochim et Biophys Acta 38 184
41 BERLINER D L & H A SALHANICK 1956 Microchemical identification of radioactive and non radioactive steroids Anal Chem 28 1608
42 BERLINER D L 1957 Microdetermination of acetylable steroids in plasma Proc Soc Exptl Biol Med 94 126
43 BERLINER D L O V DOMINGUEZ & G WESTENSKOW 1957 Determination of C[14] steroids on paper chromatograms Anal Chem 29 1797

44 GLENN F M R O STAFFORD S C LYSTER & B J BOWMAN 1957 Relation between biological activity of hydrocortisone analogues and their rates of inactivation by rat liver enzyme systems Endocrinology 61 128–142
45 DOUGHERTY T F Unpublished results
46 BUSH I E & V B MAHESH 1959 Metabolism of 11-oxygenated steroids 2 2 Methyl steroids Biochem J 71 718–742
47 DULIN N L B J BAUMAN & P O STAFFORD 1957 Effects of 2 methylation on glucocorticoid activity of various C 21 steroids Proc Soc Exptl Biol Med 94 303
48 TALALAY P & H G WILLIAMS ASHMAN 1958 Activation of hydrogen transfer between pyridine nucleotides by steroid hormones Proc Natl Acad Sci U S 44 15
49 BYERS S O M FRIEDMAN & F MICHAELIS 1951 Observations concerning the production and excretion of cholesterol in mammals J Biol Chem 188 637
50 BYERS S O 1958 Regulation of plasma cholesterol *In* Liver Function 343 351 R W Brauer Ed Am Inst Biol Sci Washington D C.
51 FRIEDMAN M S O BYERS & R H ROSENMAN 1954 Observations concerning the production and excretion of cholesterol in mammals XII Demonstration of the essential role of the hepatic reticuloendothelial cell (Kupffer cell) in the normal disposition of exogenously derived cholesterol Am J Physiol 177 77
52 NIVEC T J BIOZZI B BENACERRAF C STIFFEL & B N HALPERN The role of the reticuloendothelial system in the blood clearance of cholesterol Am J Physiol In press
53 DAOST R 1958 The cell population of liver tissue and the cytological reference bases *In* Liver Function 3-10 R. W Brauer Ed Am Inst Biol Sci Washington D C.
54 ROUS P & J W BEARD 1934 Selection with the magnet and cultivation of reticulo endothelial cells (Kupffer cells) J Exptl Med 69 577–591
55 ENGEL F L J FREDERICKS F LOPEZ & T ALBERTSON 1958 Some extra adrenal actions of corticotropin on carbohydrate metabolism in the rat Endocrinology 63 768–777
56 YATES F E J URQUHART & A I HERBST 1958 Effects of thyroid hormones on ring A reduction of cortisone by liver Am J Physiol 195 373–380

SKIN WINDOWS AND THE ACTION OF THE RETICULOENDOTHELIAL SYSTEM IN MAN

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Credit for the orderly description of a general defense system of cells divided into hematogenous (lymphocytes and large mononuclears) and histogenous macrophages belongs to Metchnikoff.[25,26] Clearer delineation of this general defense system was afforded by Aschoff and Kiyono,[1,2,13] and only those cells were included that were found to have stored vital dyes. The latter authors excluded the lymphocytes from their scheme of the reticuloendothelial system because, under the conditions of their experiments on such a system of phagocytes, they were uncertain as to whether lymphocytes had an affinity for the vital dyes. However, Tschaschin,[4,43] Downey,[8,9,10] Wierling,[41] Maximow,[21] Seki[35] and Dougherty[7] in a long series of experiments demonstrated that lymphocytes in inflammatory lesions and in tissue culture were capable of ingesting and storing such dyes until their cytoplasmic content was indistinguishable from Aschoff's ordinary histiocytes. The foregoing investigations were performed on the system of phagocytes found in laboratory animals. Hitherto the essentially certain identification afforded by vital staining has been replaced by the comparison of the structure of vital dye storing cells in animals with cells in similar situations in human tissues not in contact with vital dyes. We have modified the method of Aschoff and Kiyono for study of those cells capable of storing their vital dyes (lithium carmine, pyrrol blue, trypan blue and trypan red) in skin windows in man, that is in small simple inflammatory skin lesions that we have described elsewhere.[33,35] The methods and result of such a study follow.

Recently Marshall[3] in his extensive text on the cytology of the reticular tissue has pointed out that coincident with Aschoff's later work on his vital staining methods, Hortega del Rio and Jimenez de Asua[15,16] using an impregnation method with silver carbonate were able to demonstrate that microglia were a histogenous source of macrophages in cerebral inflammation and injury (for the further historical development of such a concept see Marshall[3]). Marshall has brought forward convincing evidence that a system of cells can be demonstrated in fixed tissues by the use of silver impregnation methods that either coincide with or overlap the reticuloendothelial system of Aschoff. The cells demonstrated by the impregnation methods of Hortega del Rio and Jimenez de Asua[15,16] and Marshall[3] are in the tissues solitary and syncytial forms of fixed metalophil cells and ameboid metalophil cells in the peripheral blood the metalophilic monocytes. Marshall, however, in section studies of sterile inflammatory lesions in rabbits claimed demarcation between lymphocytes that never showed an affinity for metallic compounds and the metalophil system of their fellow histiocytes or macrophages and monocytes; however, he did state that the large mononuclears of inflammation were admittedly to a great extent of hemic origin. In the studies reported below

Marshall's techniques were applied to the progressive influx of mononuclears in human skin windows that permit precise cytological comparison of exudative and peripheral blood cells. Lymphocytes were strongly metalophilic at 9 and 12 hours of inflammation in man, and hypertrophied lymphocytes were similarly metalophilic at 14 hours of inflammation.

Materials and Methods

The skin window technique[11, 17, 22] for studying cells from inflammatory lesions was used to obtain the specimens in this study. This technique consists of denuding a circle of the papillary layer of the corium 3 mm in diameter and observing the migration of exudative cells onto small coverslips placed over the denuded area on the volar surface of the forearm. Each lesion was challenged at 0 hour with 0.05 ml of the challenging substances (typhoid vaccine or 1:1000 tuberculin OT). The cells of the inflammatory exudate migrated to the undersurface of the coverslip. The coverslip was removed at 3 hours and replaced by another until the end of the observation period at 24 or 25 hours. Twelve lesions were prepared on 6 normal individuals; the sterile coverslips were changed at 3-, 6-, 9-, 12-, 13- or 14-hour intervals and the studies were terminated at 24 or 25 hours. Seven of the lesions were used in the study of the vital dyes (FIGURES 1 and 2). Coverslips from the remaining 5 lesions were used in the silver impregnation studies (FIGURES 3 and 4).

The vital dyes used in this study—lithium carmine (Harleco), pyrrol blue (National Aniline), trypan blue (National Aniline C.I. 477) and trypan red (National Aniline C.I. 438)—were prepared as aqueous solutions of 5.0 gm per cent, 0.5 gm per cent, 2.5 gm per cent and 2.5 gm per cent, respectively. The solutions were filtered before use. The dyes were added to the lesions at the 3 hour stage using a sterile platinum loop; the coverslips, when removed, were air-dried and stained with Leishman's stain for 12 min.

The silver impregnation technique outlined by Marshall[23] *and by Black and* Speer[3] was used with the following modifications: the air-dried coverslips were exposed to formaldehyde vapor for 15 min to fix the cells, washed in distilled water before and after the ammoniacal silver bath and stained with Leishman's stain for 15 min following the reduction of the silver and thorough washing with distilled water. To date we have been unable to apply the silver impregnation technique satisfactorily to the peripheral blood smear itself.

Results

Vital Dyes

Lithium carmine. In the experimental inflammatory lesion illustrated in FIGURE 1 less than 0.05 ml of 5.0 gm per cent aqueous solution of lithium carmine was applied with a sterile loop to the lesion at 6 hours after it had been produced and challenged with a small amount of diphtheria toxoid as described above. At 9 hours of inflammation an abundant cellular exudate was obtained that presented more than the normal number of neutrophilic leukocytes but that contained some migrant lymphocytes and a few macrophages. Only a few of the lymphocytes had stored lithium carmine at this time. At 12 hours

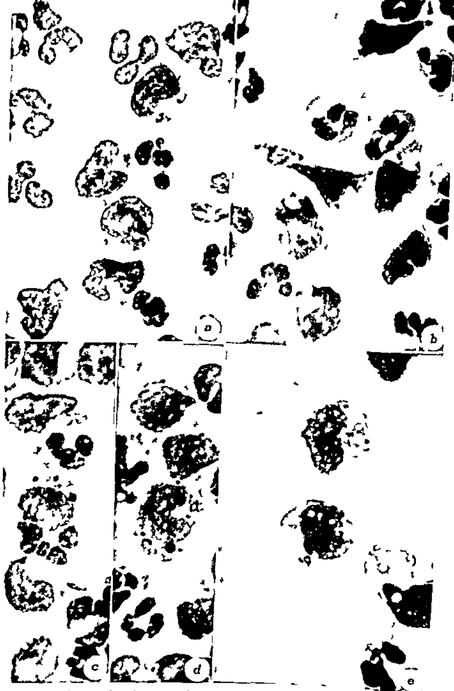


FIGURE 1 (*a*) Four lymphocytes and some neutrophils in the lesion of a normal male in the twelfth hour of inflammation. The 3 central lymphocytes contain concentrates of lithium carmine. ×1100. (*b*) Five lymphocytes and some neutrophils in the same lesion as in *a* in the fourteenth hour of inflammation. The ameboid lymphocyte and the lymphocyte to its right have both phagocytosed lithium carmine. ×1100. (*c*) Same lesion as in *a* in the fourteenth hour of inflammation. The central hypertrophied lymphocyte contains concentrates of lithium carmine. (*d*) Same lesion as in *a* and *c*. The central monocyte, which contains lithium carmine concentrates, is to be distinguished from the adjacent lymphocytes. ×1100. (*e*) Same lesion as in *a*, *c* and *d*. The top macrophage is phagocytic for lithium carmine. ×1100.

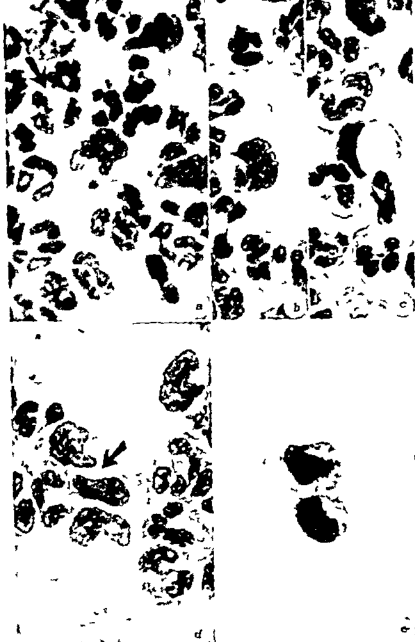


FIGURE 2 (a) Three lymphocytes and numerous neutrophils in the lesion of a normal female in the sixth hour of inflammation. The arrow points to a neutrophil that has ingested pyrrol blue. ×1100. (b) Same lesion as in a at sixth hour of inflammation. Central lymphocyte contains mass of pyrrol blue. ×1100. (c) Same lesion as in a at thirteen hours of inflammation. Central lymphocyte is ingesting a neutrophil. Fine granular pyrrol blue concentrates are seen in the thin encircling cytoplasmic rim of this lymphocyte. ×1100. (d) Two lymphocytes, 2 hypertrophied lymphocytes and some neutrophils in the lesion of a normal female in the twelfth hour of inflammation. The arrow points to a lymphocyte that has ingested trypan blue. ×1100. (e) Two slightly hypertrophied lymphocytes in the lesion of a normal female in the twelfth hour of inflammation. The top lymphocyte has ingested trypan red. ×1100



FIGURE 3 (*a* and *b*) A lymph cyte with some neut ophils i th l sion of a normal femal in the ninth hour of inflammation Each lymphocyte sho s mode ate to marked silver im pregnation ×1100 (*c*) Same le ion and time as in *a* and *b* l th amel id and roun l central lymphocytes show silver impregnation ×1100 (*d*) Fiv l n phocytes and 3 hyper trophied lymphocytes in the same le ion as in *a* *b* and *c* Seven of th 8 lymphocytes sh silver impregnation ×1100 (*e*) Three hypertrophied lymphocyt a lymphocyte and some degenerating neutrophils in the same lesion as in *a* *b* *c* and *d* All the lymphocytes are metalophil cells ×1100

the cellular exudate again contained an unusually large number of neutrophils but now many lymphocytes were present, as well as a few macrophages. FIGURE 1*a* depicts 4 such lymphocytes and some of the neutrophils at this stage. The 3 central lymphocytes show varying amounts of granular storage of the

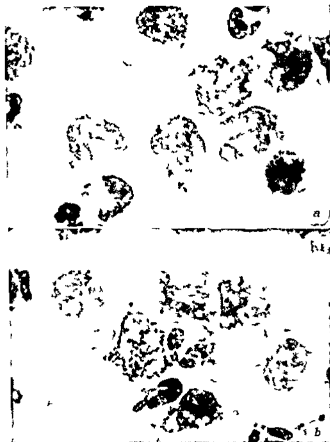


FIGURE 4. (*a*) Four monocytes and 3 lymphocytes and some degenerating neutrophils in the lesion shown in FIGURE 3. Both monocytes and lymphocytes are metalophil cells. The 4 central monocytes with their horseshoe shaped nuclei and large cell bodies are to be distinguished from the lymphocytes shown here and in FIGURE 3. ×1100. (*b*) Six macrophages and some degenerating neutrophil in the same lesion as in FIGURE 3*a* and *b*. Note that the macrophages are also metalophilic. ×1100.

dye. Note also the accentuation of the nuclear indentation of one of the lower small lymphocytes which is without granules. At 14 hours the still abundant exudate now contained many lymphocytes (FIGURE 1*b*) and their hypertrophying forms (FIGURE 1*c*) as well as additional macrophages (FIGURE 1*e*) and an occasional monocyte (FIGURE 1*d*). The neutrophils are less numerous and present their customary signs of cytoplasm loss and nuclear shrinkage for this

stage. FIGURE 1*b* presents a central ameboid lymphocyte and a small lymphocyte to its right, which have both stored lithium carmine in a granular form. FIGURE 1*c* contains a central typical hypertrophied lymphocyte with slightly more cell body than lymphocytes as found in the blood; it has concentrated 3 large masses of the lithium carmine. FIGURE 1*d* depicts similar storage of the dye by the central monocyte with its horseshoe-shaped nucleus and larger amount of cytoplasm. Typical macrophages are shown in FIGURE 1*d*. The one at the top presents a particular affinity for the carmine. At 24 hours the abundant exudate consisted largely of macrophages, although some lymphocytes and hypertrophied lymphocytes were still present as a hematogenous source of the former. These mononuclears (not illustrated) presented again some modest storage of the lithium carmine. Two additional lesions were studied after application of lithium carmine at 3 or 6 hours, and similar storage of the dye was attained by the lymphocytes, hypertrophied lymphocytes and macrophages of the exudate.

Pyrrol blue. In the experimental inflammatory lesion illustrated in FIGURE 2 less than 0.05 ml. of 0.5 gm. per cent aqueous solution of pyrrol blue was applied with a sterile loop at 3 hours after the lesion had been produced and challenged with diphtheria toxoid. At 6 hours of inflammation numerous neutrophilic leukocytes were present, but only a few lymphocytes and macrophages. The macrophages, since they were fully formed, were presumably of tissue origin. Some of the neutrophils (FIGURE 2*a*, arrow) had ingested large pale blue globules containing the pyrrol blue. The occasional lymphocytes already migrated at this stage had ingested similar blue dye-containing masses (FIGURE 2*b*), although lymphocytes with more granular storage were also apparent (central area of FIGURE 2*a*). By 12 hours there were more macrophages, a few lymphocytes and hypertrophied lymphocytes, and an unusual retention of neutrophilic predominance. At 13 hours the lymphocytes and their hypertrophied forms had increased somewhat, but still there were numerous neutrophils present (FIGURE 2*c*). The central lymphocyte in FIGURE 2*c* was fixed while ingesting a neutrophilic leukocyte; fine granular pyrrol blue concentrates were visible in the thin encircling cytoplasmic rim of this lymphocyte. At 24 hours the customary predominance of macrophages had been attained.

Trypan blue. In the experimental inflammatory lesion illustrated in FIGURE 2*d* less than 0.05 ml. of 2.5 gm. per cent aqueous solution of trypan blue was applied with a sterile loop at 3 hours of inflammation after the lesion had been produced and challenged with diphtheria toxoid. By 9 hours the numerous neutrophils of the earlier exudate had been reinforced by some lymphocytes and macrophages which showed granular concentrates of the trypan blue in their cell bodies similar to that depicted for lithium carmine in FIGURE 1*a* and *e*. At 12 hours there was again an abnormal predominance of neutrophils with some macrophages; a few lymphocytes and hypertrophied lymphocytes complete the picture. In FIGURE 2*d*, which depicts a sample of the cell at this stage, the arrow points to a lymphocyte that has ingested a trypan blue globular mass. At 14 hours lymphocytes, hypertrophied lymphocytes and macrophages had increased to form the 24-hour exudate of macrophages; moderate storage was exhibited by members of each of these mononuclear

groups A second lesion in this group was characterized by the customary granular concentrates of the dye in the small lymphocytic cell bodies

Trypan red In the experimental inflammatory lesion depicted in FIGURE 2*e* less than 0 05 ml of 2 5 gm per cent solution of trypan red was applied with a sterile loop to the lesion at 3 hours after the lesion had been produced and challenged The few lymphocytes and microphages present at 6 hour among the numerous neutrophils already showed good storage of the vital dye By 12 hours *lymphocytes hypertrophied lymphocytes and a few monocytes were* added in appreciable numbers to the neutrophils Two slightly hypertrophied lymphocytes from this lesion are depicted in FIGURE 2*e* The top lymphocyte shows the customary granular concentrates of dye storage Moderate storage by these mononuclear cell types with the addition of macrophages was found at 14 hours with termination of the study at 24 hours

Silver impregnation In the experimental inflammatory lesion depicted in FIGURES 3 and 4 the lesion was challenged by application of 0 05 ml diphtheria toxoid to its surface immediately after the lesion had been produced Without further interpolation coverslip samples of the exudate were obtained at 6 9 12 14 and 25 hours Silver impregnation studies followed by counterstaining with Leishman s stain as described under MATERIALS AND METHODS above were made on each of the preparations At 6 hours the moderate amount of exudate contained neutrophils and a few lymphocytes Even at this early stage the lymphocytes showed some silver impregnation At 9 hours more lymphocytes and hypertrophied lymphocytes together with a few monocytes had joined the numerous neutrophils In FIGURE 3*a* the small round lymphocyte in the center reveals the beginning of silver impregnation FIGURE 3*b* depicts a stronger positivity in the medium sized lymphocyte (*top cell*) In the same lesion both ameboid and round small lymphocytes show silver impregnation in the center of FIGURE 3*c* and are thus metalophils At 12 hours the abundant exudate was composed of many lymphocytes some hypertrophied lymphocytes a few monocytes as well as macrophages and some persisting neutrophils Seven of the 8 lymphocytes in FIGURE 3*d* taken at 12 hours of inflammation show silver impregnation At 14 hours of inflammation the still abundant exudate contains many lymphocytes and hypertrophied lymphocytes as well as neutrophils and macrophages and a few monocytes The metalophils are formed by each of the mononuclear cell types the lymphocytes and hypertrophied lymphocytes depicted in FIGURE 3*e* the central monocytes and peripheral lymphocytes of FIGURE 4*a* and the macrophages themselves in FIGURE 4*b* At 25 hours the hypertrophied lymphocytes and resultant macrophages were similarly silver impregnated

DISCUSSION

Downey[9] demonstrated that the lymphocytes of the blood of rats were capable of storing vital dyes if the dyes were first made available to them when the blood vessel that contained the lymphocytes had been isolated from the general circulation or if the lymphocytes were in the environment of the inflammatory field He concluded that both the time at which the foreign substance was in contact with the lymphocyte as well as the local chemical and

physical condition were of importance for storage. Under such conditions even neutrophilic leukocytes will store the vital dyes if they are similarly made available, a finding that has been confirmed for the neutrophilic leukocytes of man in our pyrrol blue experiments described above. In the first portion of our experiments described above, the lymphocytes of man similarly were phagocytic for the vital dyes pyrrol blue, lithium carmine, trypan blue, and trypan red. The phagocyte takes the vital stain only after the stain has been bound to protein, a process reviewed by Jaffe[17] in which vital staining is due to direct stimulation of an intracellular process. The dye first appears in the lymphocyte in the form of pale stained droplets (FIGURE 2b and d); thereupon the staining of the droplets increases to a point at which the dye finally precipitates as granular concentrates (FIGURE 1a and b). To gain some impression of the cellular processes required to visualize even moderate phagocytosis, it is well to consider the experiments of Lüdentz[20] on the phagocytosis of India ink. Ludentz found that India ink particles were roughly 300 Å in size, and that between 200 and 300 such particles were needed to make up an aggregate visible in the light microscope. Although it is important to demonstrate that the relatively small cell body of the human lymphocyte is capable of storage of the vital dyes, it is of much greater import for the full understanding of the concept of the reticuloendothelial system to appreciate the fact that the lymphocyte in the field of inflammation is capable of transforming into a larger and larger mononuclear until the fully formed macrophage stage is attained. It is interesting in this connection to note that even Kiyono in his later work with Nakanomin[19] admitted that after migration a few lymphocytes may transform into carmine-storing macrophages in their experimental animal. More recently Trowell[11] noted in lymph node cultures in his synthetic medium that as most of the small lymphocytes hypertrophied, their nuclear pattern became less condensed and the cells segregated trypan blue. In a previous report[35] we listed the numerous structural and chemical nuclear and cytoplasmic changes within the individual lymphocyte as hypertrophy and transformation to the macrophage state were accomplished. From the above experiments it is now obvious that the lymphocytes of man (FIGURES 1a and b and 2b, c and d), their hypertrophied forms (FIGURES 1c and 2e) and their resultant macrophages (FIGURE 1e) are all capable of storage of the vital dyes.

Application of the skin window technique to the study of experimental inflammatory lesions in man by Braunsteiner et al.,[7] Fitzman and Smith,[1,13] Kravit and Good,[1] Page and Good,[23] Priest et al.,[3] Rebuck and LoGrippo,[24] Riis[27] and Torre and Leiken[40] has now yielded far reaching confirmatory evidence that the lymphocytes of man are indeed capable of transforming into macrophages. Fichtelius[14] has observed the transformation of radioactively labeled lymphocytes to macrophages in peritoneal exudate.

Elsewhere[35] we have followed the transformation of individual supravitally stained lymphocytes from cantharides blisters as they transformed into phagocytic supravital monocytes. Recently Klein[9] has reported following similar transformations of lymphocytes to macrophage, back to lymphocytes and retransformation to macrophages by phase-contrast microscopy in microcinematography. We now add the results of our second group of experiments re-

ported above, namely, the demonstration that the lymphocytes (FIGURE 3*a*, *b* and *c*) in the field of inflammation in man are metalophil in the sense of Hortega del Rio[16] and of Marshall,[23] and as such are capable of transformation to hypertrophied lymphocytes (FIGURE 3*d* and *e*) and macrophages (FIGURE 4*b*) that are similarly metalophilic. Marshall,[23] by performing metallic impregnations upon the tissues of vitally stained animals, has shown that in many sites the impregnated cells are the same as those that contain the vital dye. Furthermore, all cells that possessed dye-storing properties qualifying them for inclusion in the reticuloendothelial system were also metalophilic, although many metalophilic cells in the pulp of the spleen and the medulla of lymph nodes in vitally stained animals, in contrast, showed little evidence of storage of vital dyes. In this respect, then, the lymphocytes in the field of inflammation in man, as lymphocytes, as they hypertrophy and, finally, as they transform into macrophages, both store vital dyes and are metalophilic.

The more precise cytological examinations yielded by recent studies of the inflammatory lesion of man through serial samplings of the leukocytic exudates has afforded further insight into the abnormalities suffered by his leukocytic defenses. Page and Good[8,9] in cyclic neutropenia and Rus[37] in severe neutropenias have both noted striking deficiencies of lymphocytes and their resultant hematogenous macrophages in the experimental skin lesions of such patients. Noting that lymphocytic participation returned when the neutrophil levels in the circulating blood and subsequent inflammatory lesions improved, Page and Good[9] were able to correlate failure of lymphocytic migration and transformation to macrophages with experimentally induced neutropenias in rabbits. As early as 1910 Opie[27] had proposed that "it is not impossible that polynuclear leukocytes together with other products of tissue degeneration serve as the principal stimulus to the activity of the mononuclear cell." Steinberg,[39] working with experimental *Escherichia coli* infections in dogs, demonstrated that the intraperitoneal injection of granulocytes increased survival rates and times, stressing the importance of neutrophilic leukocytes in the early phase of inflammation. In an earlier report[33] we described in detail the progressive depletion of neutrophilic cytoplasm and the ingestion of shed neutrophilic leukocytic debris by the lymphocytes as they hypertrophied and were subsequently transformed into macrophages. Recently, Policard and his colleagues[30] have studied, with the electron microscope, the predegenerative processes of the neutrophilic leukocytes in the field of aseptic inflammation and have observed rapid loss of specific granulation, hyperplasia and hyperactivity of endoplasmic reticulum and dilatation of the Golgi apparatus at the ultrastructural level.

Individual assessment of leukocytic defenses in the leukemias and allied disorders[35,37,40] has revealed wide variations, ranging from normal leukocytic cycles to almost complete lack of leukocytic migrations.

Similarly, increased eosinophilic migrations in human skin windows[34,37,40] have been associated with increased or normal peripheral blood eosinophilic levels. Similarly, high blood eosinophilias have been associated with increased or normal eosinophilic migrations into the field of inflammation. Of particular interest are the reports of Eitzman and Smith[12,13] of an increase in eosinophils in

physical conditions were of importance for storage. Under such conditions even neutrophilic leukocytes will store the vital dyes if they are similarly made available, a finding that has been confirmed for the neutrophilic leukocytes of man in our pyrrol blue experiments described above. In the first portion of our experiments described above the lymphocytes of man similarly were phagocytic for the vital dyes pyrrol blue, lithium carmine, trypan blue and trypan red. The phagocyte takes the vital stain only after the stain has been bound to proteins, a process reviewed by Jaffe[17] in which vital staining is due to direct stimulation of an intracellular process. The dye first appears in the lymphocyte in the form of pale stained droplets (FIGURE 2b and d); thereupon the staining of the droplets increases to a point at which the dye finally precipitates as granular concentrates (FIGURE 1a and b). To gain some impression of the cellular processes required to visualize even moderate phagocytosis it is well to consider the experiments of Lüderitz[20] on the phagocytosis of India ink. Lüderitz found that India ink particles were roughly 300 Å in size and that between 200 and 300 such particles were needed to make up an aggregate visible in the light microscope. Although it is important to demonstrate that the relatively small cell body of the human lymphocyte is capable of storage of the vital dyes, it is of much greater import for the full understanding of the concept of the reticuloendothelial system to appreciate the fact that the lymphocyte in the field of inflammation is capable of transforming into a larger and larger mononuclear until the fully formed macrophage stage is attained. It is interesting in this connection to note that even Kiyono in his later work with Nakanoin[19] admitted that after migration a few lymphocytes may transform into carmine storing macrophages in their experimental animal. More recently Trowell[38] noted in lymph node cultures in his synthetic medium that as most of the small lymphocytes hypertrophied, their nuclear pattern became less condensed and the cells segregated trypan blue. In a previous report[35] we listed the numerous structural and chemical nuclear and cytoplasmic changes within the individual lymphocytes as hypertrophy and transformation to the macrophage state were accomplished. From the above experiments it is now obvious that the lymphocytes of man (FIGURES 1a and b and 2b, c and f), their hypertrophied forms (FIGURES 1c and 2e) and their resultant macrophages (FIGURE 1e) are all capable of storage of the vital dyes.

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8 Downey H 1916-1917 Histiocytes and macrophages and their relations to the cells of normal blood in animals stained with acid colloidal dyes intravitam Anat Record 11 350
9 Downey H 1917 Reactions of blood and tissue cells to acid colloidal dyes under experimental conditions Anat Record 12 429
10 Downey H 1918 Further studies on the reactions of blood and tissue cells to acid collo dal dyes Anat Record 15 103
11 Edwards J L 1959 Cytology of inflammatory exudate Lab Invest 8 1091
12 Fitzman D V & R T Smith 1957 The nonspecific inflammatory response in the newborn period J Diseases Children 94 494
13 Fitzman D V & R T Smith 1959 The nonspecific inflammatory cycle in the neonated infant J Diseases Children 97 326
14 Fichtelius K E cited by H Braunsteiner[4]
15 Hortega del Rio L & F Jimenez de Asua 1921 Sobre la fagocitosis de los tumores y en otros procesos patológicos Arch ca di Haematol 2 161
16 Hortega del Rio P & F Jimenez de Asua 1921 Sobre las cellulas del reticulo esplenico y sus relaciones con el endothelio sinusal Bol Real Soc espan Hist Nat An 11 7
17 Jaffé R H 1938 The reticulo-endothelial system *In* Handbook of Hematology II 973 H Downey Ed Hoeber New York N Y
18 Kiyono K 1914 Die vitale Karminspeicherung Fischer Jena Germany
19 Kiyono K & T Nakanoin 1919 Weiterer untersuchungen über die histiozytären Zellen Acta scholae med Univ Imp in Kioto 3 55
20 Klein R 1959 Demonstration par la microcinematographie en contraste de phase de la transformation reversible des lymphocytes en macrophages *in vitro* Compt rend acad soc biol 153 545
21 Krivit W & R A Good 1959 Aldrich s syndrome (thrombocytopenia eczema and infection in infants) Studies of the defense mechanisms J Diseases Children 97 137
22 Luderitz B 1957 Intracellulare Tuschespeicherung in pathologisch vergrösserten Lymphdrusen Acta Haematol 17 143
23 Marshall A H F 1956 An outline of the cytology and pathology of the reticular tissue Thomas Springfield Ill
24 Maximow A 1928 Cultures of blood leukocytes From lymphocyte and monocyte to connective tissue Arch exptl Zellforsch Gewebezucht 5 169
25 Metchnikoff E 1893 Lectures on the comparative pathology of inflammation (1891) Translated by F & E Starling Kegan Paul Trench & Trubner London England
26 Metchnikoff E 1905 Immunity in infective disease Translated by F G Binnie Univ Press Cambridge England
27 Opie E L 1910 Inflammation Arch Intern Med 5 541
28 Page A R & R A Good 1957 Studies on cyclic neutropenia J Diseases Children 94 623
29 Page A R & R A Good 1958 A clinical and experimental study of the function of neutrophils in the inflammatory response Am J Path l 34 645
30 Policard A A Collet & S Prégermain 1959 Étude en microscope électronique des modifications infrastructurales présentées par les polynucleaires neutrophiles dans des foyers inflammatoires aseptiques Rev Hematol 14 97
31 Priest R J J W Rebuck & G T Havey A new qualitative defect of leukocyte function in ulcerative colitis Gastroenterology In press
32 Rebuck J W 1958 Technic for the study of leukocytic functions in man *In* Methods in Medical Research Warren Year Book Publ Chicago Ill
33 Rebuck J W & J H Crowley 1955 A method of study of leukocytic functions *N Y Ac* Ann N Y Acad Sci 59(5) 757
34 Rebuck J W & C A LoGrippo Leukocytic responses to poliomyelitis vaccines in human skin windows Proc 3rd Intern Symposium on Diseases of the Reticulo Endothelial System Ronald Press New York N Y In press
35 Rebuck J W R W Monto F A Monaghan & J M Riddle 1958 Potentialities of the lymphocyte with an additional reference to its dysfunction in Hodgkin s disease Ann N Y Acad Sci 73(3) 8
36 Rebuck J W R W Smith & R P Margulis 1951 The modifications of leukocytic function in human windows by ACTH Gastroenterology 19 644
37 Riis P 1959 The Cytology of Inflammatory Exudate Munksgaard Copenhagen Denmark

the inflammatory lesions averaging 19 per cent of the exudate cells in infants 2 to 21 days of age 2 hours after the initiation of the lesion which was not seen in infants less than 24 hours of age or in older children and was out of proportion to their circulating blood eosinophil levels

Priest and his associates[31] have similarly reported an increased migration of basophilic leukocytes into the inflammatory lesions of patients with ulcerative colitis in whom blood basophil levels were not elevated This was accompanied by a variation in the pattern of their exudative cytology, which differed from the normal by failure of the early predominance of neutrophils to yield to an adequate lymphocytic exudation

It is apparent that we are entering a period in which mechanisms and abnormalities of the process of lymphocytic transformation into macrophages will find further clarification although furnishing newer problems concerning granulocyte mononuclear and granulocyte granulocyte interrelationships

Summary

Aschoff in his extension of Metchnikoff's general defense system of cells, included only those cells that were found to have an affinity for vital dyes Since his methods were applied to the study of such a system of phagocytes in laboratory animals we modified his method for study of those cells phagocytic for the same vital dyes (pyrrol blue lithium carmine trypan blue and trypan red) in skin windows in man In such inflammatory lesions lymphocytic hypertrophied lymphocytes macrophages and monocytes all showed an ability to store the above listed vital dyes

Recently Marshall claimed demarcation in sterile inflammatory exudates in rabbits between lymphocytes that could not be impregnated with silver and metalophil system of their fellow histiocytes or macrophage and monocytes Applying his techniques to the progressive influx of mononuclears in human skin windows lymphocytes were strongly metalophilic at 9 and 12 hours of inflammation in man and hypertrophied lymphocytes were similarly metalophilic at 14 hours of inflammation

The importance of the granulocytic interrelationships with proper RES functions in these lesions was emphasized by correlation of low neutrophilic participation with low lymphocytic responses and increased basophilic leukocytic migrations with impaired mononuclear defense

References

1 Aschoff L 1924 Lectures on Pathology Hoeber New York N Y

2 Aschoff L & K Kiyono 1913 Zur Frage der grossen Mononukleären [illegible] Haematol 15 383

3 Black M M & F D Speer 1958 Antigenic [illegible] changes in lymph nodes [illegible] lophilia A M A Arch Pathol 66 754

4 Braunsteiner H 1959 Physiologie und Physiopathologie der weissen Blutzellen 79–83 Thieme Stuttgart Germany

5 Braunsteiner H J Partan & N Thumb 1957 Function of the lymphocyte J Am Med Assoc 164 1604

6 Braunsteiner H J Partan & N Thumb 1958 Studies on lymphocytic function Blood 8 417

7 Dougherty T F 1944 Studies on the cytogenesis of microglia and their relation to cells of the reticuloendothelial system Am J Anat 74 61

8 Downey H 1916-1917 Histiocytes and macrophages and their relations to the cells of normal blood in animals stained with acid colloidal dyes intravitam Anat Record 11 350
9 Downey H 1917 Reactions of blood and tissue cells to acid colloidal dyes under experimental conditions Anat Record 12 429
10 Downey H 1918 Further studies on the reactions of blood and tissue cells to acid colloidal dyes Anat Record 15 103
11 Edwards J L 1959 Cytology of inflammatory exudate Lab Invest 8 1091
12 Fitzman D V & R T Smith 1957 The nonspecific inflammatory response in the newborn period J Diseases Children 94 484
13 Eitzman D V & R T Smith 1959 The nonspecific inflammatory cycle in the neonated infant J Diseases Children 97 326
14 Fichtelius K E *cited by* H Braunsteiner[4]
15 Hortega del Rio L & F Jimenez de Asua 1921 Sobre la fagocitosis de los tumores y en otros procesos patológicos Arch card ol Haematol 2 161
16 Hortega del Rio P & F Jimenez de Asua 1921 Sobre las cellulas del reticulo esplenico y sus relaciones con el endothelio sinusal Bol Real Soc espan Hist Nat An 11 7
17 Jaffé R H 1938 The reticulo-endothelial system *In* Handbook of Hematology II 973 H Downey Ed Hoeber New York N Y
18 Kiyono K 1914 Die vitale Karminspeicherung Fischer Jena Germany
19 Kiyono K & T Nakanoin 1919 Weiterer untersuchungen uber die histiozytären Zellen Acta scholae med Univ Imp in Kioto 3 55
20 Klein R 1959 Demonstration par la microcinematographie en contraste de phase de la transformation reversible des lymphocytes en macrophages *in vitro* Compt rend acad soc biol 153 545
21 Krivit W & R A Good 1959 Aldrich s syndrome (thrombocytopenia eczema and infection in infants) Studies of the defense mechanisms J Diseases Children 97 137
22 Luderitz B 1957 Intracellulare Tuschespeicherung in pathologisch vergrosserten Lymphdrusen Acta Haematol 17 143
23 Marshall A H F 1956 An outline of the cytology and pathology of the reticular tissue Thomas Springfield Ill
24 Maximow A 1928 Cultures of blood leukocytes From lymphocyte and monocyte to connective tissue Arch exptl Zellforsch Gewebezücht 5 169
25 Metchnikoff F 1893 Lectures on the comparative pathology of inflammation (1891) Translated by F & F Starling Kegan Paul Trench & Trubner London England
26 Metchnikoff F 1905 Immunity in infective disease Translated by F G Binnie Univ Press Cambridge England
27 Opie F L 1910 Inflammation Arch Intern Med 5 541
28 Page A R & R A Good 1957 Studies on cyclic neutropenia J Diseases Children 94 623
29 Page A R & R A Good 1958 A clinical and experimental study of the function of neutrophils in the inflammatory response Am J Pathol 34 645
30 Policard A A Collet & S Prégermain 1959 Étude en microscope électronique des modifications infrastructurales presentées par les polynucleaires neutrophiles dans des foyers inflammatoires aseptiques Rev Hematol 14 97
31 Priest R J J W Rebuck & G T Havey A new qualitative defect of leukocyte function in ulcerative colitis Gastroenterology In press
32 Rebuck J W 1958 Techniques for the study of leukocytic functions in man *In* Methods in Medical Research Warren Year Book Publ Chicago Ill
33 Rebuck, J W & J H Crowley 1955 A method of study of leukocytic functions *in vivo* Ann N Y Acad Sci 59(5) 757
34 Rebuck J W & G A LoGrippo Leukocytic responses to poliomyelitis vaccines in human skin windows Proc 3rd Intern Symposium on Diseases of the Reticulo-Endothelial System Rome Pless New York N Y In press
35 Rebuck J W R W Monto F A Monaghan & J M Riddle 1958 Potentialities of the lymphocyte with an additional reference to its dysfunction in Hodgkin s disease Ann N Y Acad Sci 73(3) 8
36 Rebuck J W R W Smith & R R Margulis 1951 The modifications of leukocytic function in human windows by ACTH Gastroenterology 19 644
37 Riis P 1959 The Cytology of Inflammatory Exudate Munksgaard Copenhagen Denmark

38 SEKI M 1935 Zur Kenntnis der intra und supravitalen Farbung IX Z Zellforsch u mikroskop Anat 23 314

39 STEINBERG B 1939 Inflammation of serous surfaces Transfer of living leukocytes and the effect on acute infectious states Arch Pathol 26 785

40 TORRE C M & S I LEIKEN 1959 Tissue inflammatory cytology in clinical states Am J Clin Pathol 32 335

41 TROWELL O A 1958 Some properties of lymphocytes *in vivo* and *in vitro* Ann N Y Acad Sci 73(3) 105

42 TSCHASCHIN S 1913 Ueber die Herkunft und Entstehungsweise der lymphozytoiden *(leukozytoiden) Zellen der Polyblasten bei der Entzundung* Folia Haematol 16 247

43 TSCHASCHIN S 1914 *Ueber die ruhenden Wanderzellen und ihre Beziehungen zu* den anderen Zellformen des Bindegewebes und zu den Lymphozyten Folia Haemtol 17 317

44 WIERLING A 1926 Experimenteller Beitrag zur Geschichte der Wanderzellen bei Amphibien Z Anat Entwicklungsgeschichte 81 448

ACTIVATION OF CAPILLARY ENDOTHELIUM*

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Parenteral administration of natural and synthetic polymers produces systemic disorders in certain species of laboratory animals. Basic and major disturbances produced by polymers have been considered as consequences of capillary damage reflected in increased permeability.[1] The intraperitoneal injection of dextran is followed by edema formation in the extremities of albino rats.[2] The alterations in the function of the capillary endothelium as well as the suspected capillary damage during the dextran induced edema were investigated in normal and histamine- or 5-hydroxytryptamine (5-HT) depleted rats. The purpose of this communication is to report our pertinent observations.

Methods and Results

Alterations in the Function of Capillary Endothelium

In order to obtain information on the behavior of the common endothelium during the dextran induced edema in the adult Sprague Dawley rats (115 to 150 gm in weight) observations have been gathered by the simultaneous use of four variants of the methodology.

(1) Intravenously injected India ink accumulates at sites of the skin that have been previously subjected to injury or stimulus.[3] Carbon accumulation like edema formation is considered a result of increased capillary permeability due to the injury.[3,4] Clinical dextran was injected intraperitoneally in a dose of 30 mg/100 gm body weight. Twice previously, at the beginning of the edema formation and at the time when edema was in decrease, Gunther's India ink in saline solution containing 2 per cent gelatin was injected into the tail vein of rats in a dose of 12 mg carbon/100 gm. The intensity of carbon accumulation (graded as 0, ±, +, ++, +++, or ++++) at the extremities was estimated at the time these animals were sacrificed.

(2) The dextran induced edema appears at the face, ears and extremities but no visible alterations occur elsewhere. To obtain information on the the behavior of the capillary endothelium at sites of the skin where no edema occurs, a stimulus was applied in the abdominal skin before and during the progress and the regression of the edema in the corresponding 3 groups of rats respectively. The animals received an intradermal injection of histamine, 5-HT or 48/80 (a polymer of N-methyl homoanisylamine and formaldehyde) in the abdominal region, the skin having been depilated the previous day by an electric razor. Immediately thereafter India ink was injected intravenously. Carbon accumulation in the abdominal skin was registered as 0, ±, +, ++, +++ and ++++ when animals were sacrificed, and readings were made at the inner side of the skin after its removal.

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(3) The clearance of India ink from the blood stream indicates the function of the reticuloendothelial system (RES) and of the common endothelium if the cells of the latter acquire a transitory storing capacity such as occurs in the case of an injury or stimulus. The carbon content of the blood was measured 4 times at 10 min intervals in the 3 groups of rats that had received India ink before and during the progress and regression of the dextran induced edema.

(4) Alterations in the capillary endothelium and in the pericapillary space were studied with a phase contrast microscope in unstained freshly prepared thin layers of the subcutis immediately *post mortem*, several preparations having been made from each animal.

The experimental set up was such that the above 4 varieties of methodology were applied simultaneously on each rat as follows:

Dextran (30 mg/100 gm) was injected intraperitoneally into each rat. India ink was injected intravenously at different time intervals after dextran (*a*) before edema formation, (*b*) during the progress of edema development, and (*c*) when the edema was fully developed.

Each group of *a*, *b*, *c* rats was divided into 3 subgroups and simultaneously with the India ink, 5 rats received intradermally 20 μg histamine bichlorhydrate, 0.2 μg 48/80 and 0.2 μg 5-HT in 0.1 ml saline respectively into the abdominal skin. After the injection of India ink, carbon content of the blood was determined by the photometric method of Heller *et al.*[6] 0008-ml samples having been taken from the tail vein at regular time interval. Intensity of edema formation was registered as a percentage of maximal intensity by the method of West at the time intervals shown in TABLES 1 to 4. Each rat was sacrificed 4 hours after dextran injection and carbon accumulation in the skin was tabulated as described above.

As shown in TABLE 1, 40 min after injection of dextran there was no edema in some rat while in others edema developed at varying intensities. At this constant time India ink was injected intravenously. Carbon particles disappeared at a normal rate from the blood of nonedematous rats at 40 min. There was an intense carbon accumulation at the extremities and also in the abdominal skin where stimuli (histamine, 5-HT or 48/80) were applied. In the other rats which showed edema formation in progress at 40 min, unusually high carbon content was measured in the blood during the initial 20 min, and carbon accumulation in any part of the skin was either absent or of but limited intensity. If India ink was injected before, during or after the edema development, the same fluctuations were observed in the behavior of the capillary endothelium as shown in TABLE 2.

The results shown in TABLES 1 and 2 can be summarized as follows:

(1) India ink accumulates at the extremities where later edema develops as well as in the skin where stimuli were used prior to the appearance of edema. At this time the removal of carbon from the blood proceeds at a normal rate.

(2) At the time when edema formation is in progress there is no deposition of India ink at the sites of edema nor at any other site.

(3) With the progressive diminishing or disappearing of the edema reactions of India ink accumulation reappear.

(4) During the presence of edema the carbon particles are retained in circulation only during the initial 20 min the carbon clearance then proceeds at a normal rate since India ink disappears within 60 to 80 min from the edematous rats just as from the normal ones

TABLE 1

Number of rats	1	2	3	4	5	6	7	8	9	10
Edema (per cent)	55	0	0	75	0	100	45	0	85	100
Carbon accumulation										
After stimulus	++	++++	++++	+	++++	ø	++	+++	+	±
Extremities	++	++++	+++	+	+++	±	+	++	+	±
	Carbon in blood μg/ml									
Minutes after India ink										
10	524	162	264	1250	236	1300	325	234	1250	1250
20	480	71	197	972	123	1050	272	178	715	578
30	299	66	125	625	108	578	233	72	474	354
40	260	25	108	278	74	276	187	68	276	225

Intensity of edema (per cent of maximum) in rats 40 min after I P injection of dextran At this time 20 μg histamine and 0 2 μg 5 HT was injected I D and India ink I V Carbon accumulation is shown in the skin of extremities and where stimuli (histamine and 5 HT) are applied The disappearance of carbon from the blood was measured at regular time intervals after the injection of India ink There was no accumulation of the ink in the skin and an unusually high amount of carbon was present in the blood of animals with fully developed edema

TABLE 2

India ink I V (min after dextran)	Edema (per cent)	Carbon accumulation		Carbon in blood (μg/ml) (min after India ink)			
		Extremities	Stimuli	10	20	30	40
30	0	++++	++++	236	123	88	46
60	100	±	±	1250	715	548	281
180	55	++	++	340	244	210	170
240	0	+++	++++	264	187	109	77

Intensity of edema (per cent of maximum) 30 60 180 and 240 min after I P injection of dextran At each time histamine and 5 HT (stimuli) were injected I D and India ink I V Carbon accumulation in the abdominal skin where histamine and 5 HT are injected and in the extremities was being registered 4 hours after dextran Carbon content of the blood was measured at regular time intervals after injection of India ink Each mark represents average readings in 5 rats

In the thin layers of the surviving subcutis the phase contrast microscope reveals the morphologic alterations in and around the capillaries during the above phenomena thus completing the observations Photomicrographs of FIGURE 1 clearly show that whenever India ink is injected before the appearance of edema carbon particles adhere to the capillary wall and appear within a short time in the pericapillary space This acquired activity of the common endothelium to accumulate to store and to become permeable for

(3) The clearance of India ink from the blood stream indicates the function of the reticuloendothelial system (RES) and of the common endothelium if the cells of the latter acquire a transitory storing capacity such as occurs in the case of an injury or stimulus. The carbon content of the blood was measured 4 times at 10 min intervals in the 3 groups of rats that had received India ink before and during the progress and regression of the dextran induced edema.

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The experimental set up was such that the above 4 varieties of methodology were applied simultaneously on each rat as follows.

Dextran (30 mg/100 gm) was injected intraperitoneally into each rat. India ink was injected intravenously at different time intervals after dextran (*a*) before edema formation (*b*) during the progress of edema development, and (*c*) when the edema was fully developed.

Each group of *a* *b* *c* rats was divided into 3 subgroups and, simultaneously with the India ink 5 rats received intradermally 20 μg histamine bichlorhydrate 0.2 μg 48/80 and 0.2 μg 5 HT in 0.1 ml saline respectively into the abdominal skin. After the injection of India ink carbon content of the blood was determined by the photometric method of Heller *et al.*[6] 0008-ml samples having been taken from the tail vein at regular time intervals. Intensity of edema formation was registered as a percentage of maximal intensity by the method of West[7] at the time intervals shown in TABLES 1 to 4. Each rat was sacrificed 4 hours after dextran injection and carbon accumulation in the skin was tabulated as described above.

As shown in TABLE 1 40 min after injection of dextran there was no edema in some rats, while in others edema developed at varying intensities. At this constant time India ink was injected intravenously. Carbon particles disappeared at a normal rate from the blood of nonedematous rats at 40 min. There was an intense carbon accumulation at the extremities and also in the abdominal skin where stimuli (histamine 5 HT or 48/80) were applied. In the other rats which showed edema formation in progress at 40 min unusually high carbon content was measured in the blood during the initial 20 min and carbon accumulation in any part of the skin was either absent or of but limited intensity. If India ink was injected before during or after the edema development the same fluctuation were observed in the behavior of the capillary endothelium as shown in TABLE 2.

The results shown in TABLES 1 and 2 can be summarized as follows.

(1) India ink accumulates at the extremities where later edema develop as well as in the skin where stimuli were used prior to the appearance of edema. At this time the removal of carbon from the blood proceed at a normal rate.

(2) At the time when edema formation is in progress there is no deposition of India ink at the sites of edema nor at any other site.

(3) With the progressive diminishing or disappearing of the edema reactions of India ink accumulation reappear.

particulate matter has been characterized by us as endothelial activation since these phenomena never occur singly. High power magnification clearly demonstrates that carbon particles accumulate on or in an endothelial cell activated by a stimulus rather than in the interstices of the cells. At any moment all capillaries and all endothelial cells are not equally susceptible to stimulation as shown also by Alksne.[8] Histology confirms the findings presented in TABLES 1 to 4, according to which no endothelial activation ensues if India ink is injected during the progress of edema. No capillary damage is observed at any instant during the above capillary alterations. The capillary wall and structure appeared to be normal before and during edema as well as after the application of histamine, 5-HT, or 48/80, but the disintegration of the capillary structure does occur if higher amounts than 0.2 µg 5-HT are injected intradermally.

Edema formation is a consequence of increased permeability; however, as soon as the outward flow of plasma fluids begins across the capillary wall the endothelial cells do not accumulate particulate matter from the blood; this is a characteristic feature of these cells at an earlier time before the outward flow begins. How far the endothelial activation and increased permeability for plasma fluids are mediated by histamine or by 5-HT are the subject of further investigations.

Alterations in the Function of the Capillary Endothelium in Histamine- and 5-HT-Depleted Rats

In this series of experiments animals were pretreated in 3 groups: (1) by depletion with reserpine[9] when 90 per cent of the 5-HT depots were emptied; (2) by treatment with polymyxin B[10] when 90 per cent of the histamine depots were depleted; and (3) by depletion of the histamine and 5-HT depots with 48/80 pretreatment.[10]

The rats thus prepared were then submitted to the experiments by the application of the methodology described above. The results presented in TABLE 3 show that:

(1) In the skin of rats subjected to any form of depletion the reaction of India ink accumulation cannot be brought about by any of the stimuli.

(2) In all 3 groups of depleted rats dextran elicits edema at the same intensity as in the normal ones.

(3) There is no carbon accumulation in the extremities or in any other part of the skin where histamine, 5-HT, or 48/80 has been injected if depleted rats received dextran.

Microscopic examinations confirmed these observations. In the depleted rats no or very few carbon particles adhered to the capillary endothelium at

FIGURE 1. Phases of the activation of capillary endothelium. Photomicrographs taken with phase-contrast microscope from unstained surviving thin layers of the subcutis. Photomicrographs *a* and *b* show adsorption of particulate matter on the capillary endothelium and storage of the carbon particles by an endothelial cell. In *c* and *d* migration of carbon particles into the pericapillary space is shown. All of these photomicrographs are taken from capillaries of the foot pads of rats treated with dextran. Capillary activation occurs before the appearance of edema, but not simultaneously with the edema formation, increased permeability being a selective function either for particulate matter or for the plasma fluids. Note that there is no capillary damage during activation.









those sites of the skin where stimulators were injected neither could any particles be discovered in the pericapillary space

Capillary Alterations as Influenced by Antihistamines

Further evidence can be obtained that endothelial activation on the one hand and increased permeability for plasma fluids on the other are entirely different features in the acute inflammatory reaction According to a previous report[11] R.P 6847 (diazo-9 9[dimethylamino-3 methyl propyl] 10 phenothiazine HCl) inhibits the capillary effects of histamine only but not those of 5 HT or 48/80 and it is known that R P 2786 (mepyramine maleate) inhibits only the accumulation of the particulate matter but does not prevent the formation of dextran induced edema[7] Animals were divided into 3 groups

The first received R P 2786 (5 mg/100 gm) intramuscularly 25 min before dextran the second received R P 6847 (5 mg/100 gm) intramuscularly 25 min before dextran and the third received saline solution intramuscularly 25 min before dextran

Each group was divided into 3 subgroups the animal having received 10 minutes after dextran respectively 20 μg histamine intradermally and India ink intravenously 0 2 μg 5 HT intradermally and India ink intravenously or 0 2 μg 48/80 intradermally and India ink intravenously

TABLE 4 shows the results obtained The dextran edema developed at full intensity even if the capillary effects of histamine were inhibited by preventive administration of R P 6847 Also edema was provoked by dextran even if the endothelial activation was completely inhibited with R P 2786

Removal of Carbon Particles from the Blood Stream

Previous experiments show that during the development of the edema the removal of particulate matter from the circulation is altered transitorily thus carbon is retained in the blood in unusually high concentration for about 20 min This altered removal can be caused (1) by the retention of particulate matter in the blood because of the absence of an effective filtration pressure[1] or by vasoconstriction (2) by inadequate function of the RES or (3) by hemoconcentration

The congestion of carbon in the blood during dextran edema cannot be explained with the fall of blood pressure since this reaches its normal level 5 to 8 min after dextran has been injected[1] At this time there is a normal rate of carbon removal from the blood therefore the carbon content in the blood is not affected by the transitory fall of the blood pressure The function of the RES during edema must be considered as normal since no difference is observed in the rate of carbon clearance between dextran treated and the normal rats in both groups the carbon disappears within 60 to 80 min It remained to determine whether the congestion of carbon in the vessels is a consequence of hemoconcentration due to the outward flow of plasma fluids during the edema To settle this question red blood cell counts were made at regular time interval after dextran injection in the rats Alterations in hemoconcentration and in carbon removal as well as the intensity

Table 3

Depletion (agent used)	Group of rats	Hours after depletion	Saline I P				Dextran 30 mg/100 gm I P								Oedema (per cent)
			Blue ink accumulation												
							Before oedema				During oedema				
			H	5 HT	48/80	Extremities	H	5 HT	48/80	Extremities	H	5 HT	48/80	Extremities	
Histamine (polymyxin B)	1	18	0	0	0	0	0	0	0	0	0	0	0	0	100
	2	48	0	++	0	±	0	++	0	0	0	0	0	0	100
	3	72	+++	++++	++++	+++	+++	++++	++++	+++	0	0	0	0	100
5 HT (reserpine)	4	18	0	0	0	0	0	0	0	0	0	0	0	0	100
	5	48	0	0	0	0	0	0	0	0	0	0	0	0	100
	6	72	++	++	++	++	++	++	++	++	0	0	0	0	100
	7	120	+++	++++	++++	+++	++++	++++	++++	+++	0	0	0	0	100
	8	170	++++	++++	++++	++++	++++	++++	++++	+++	0	0	0	0	100
Histamine + 5 HT (48/80)	9	18	0	0	0	0	0	0	0	0	0	0	0	0	100
	10	48	0	++	0	+	0	++	0	±	0	0	0	0	100
	11	72	+++	+++	++++	+++	+++	+++	++++	+++	0	0	0	0	100
Normal (saline)	12		++++	++++	++++	++++	++++	++++	++++	++++	0	0	0	0	100

Intensity of oedema (per cent of maximum) in histamine and/or 5 HT depleted rats when dextran was injected at different time intervals after depletion was completed Histamine (H) 5 HT or 48/80 was injected I D India ink I V before the appearance of edema and during edema respectively Carbon accumulation in the abdominal skin where stimulators were used as well as in the extremities are registered after the animals were sacrificed (4 hours after dextran) The same reactions are shown in rats having received saline instead of dextran Each mark represents average readings in 5 rats

time plasma fluids can freely flow out into the pericapillary space of the damaged area Identification of the suspected mediators has been approached in further experiments on depleted rats with the help of antihistamines

Carbon Removal in Depleted Rats as Influenced by Antihistamines

Experiments were performed on normal as well as on histamine and 5 HT depleted rats In all animals the removal of carbon from the blood was determined as above Antihistamines (derivatives of phenothiazine) were injected 25 min before dextran

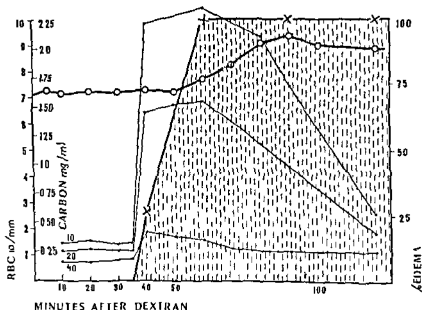


FIGURE 2 Intensity of edema X——X (per cent of maximum) number of red blood cells O——O (10 /mm³ in blood) and carbon content in blood ——• (mg /ml) of dextran treated rats Carbon content in blood is measured 10 20 and 40 min after I V injection of India ink Dots in graph ——• represent time when India ink has been injected Average readings in 10 rats

Results presented in FIGURE 3 show that

(1) The carbon particles are removed from the blood stream at a normal rate if the animals are treated preventively prior to dextran with R P 3277 (promethazine 10 mg /100 gm) R P 4560 (chlorpromazine 2 5 mg /100 gm) and R P 7044 (levopromazine 750 µg /100 gm) It is known that these phenothiazines inhibit edema formation and the reaction of carbon accumulation as well

(2) Carbon particles are retained in the vessels during the presence of edema when rats are pretreated with R P 2786 (2 mg /100 gm) and R P 6847 (5 mg /100 gm)

(3) The rate of carbon removal from the blood of rats depleted of his

of the edema are shown in FIGURE 2. Carbon particles are retained at extremely high concentration in the blood during the first 20 min. when edema formation is developing. At this time there is but slight elevation of the RBC figures. The intensity of the edema remains high for the next hour, the maximal hemoconcentration being reached 30 to 40 min. after the edema has started to develop. As soon as the hemoconcentration attains its peak the carbon removal is again nearly normal.

It is of particular interest that the congestion of the carbon particles lasts for but a short time and this is exactly the period during which the increased

TABLE 4

Stimuli	Pretreatment	30 mg/100 gm dextran I.P.						Saline I.P.
		India ink (min. after dextran)						India ink
		30	60	90	120	180	240	
Edema (per cent)								
	R.P. 2786	—	100	100	75	50	0	0
	R.P. 6847	—	100	100	70	50	0	0
	Saline	—	100	100	70	50	0	0
India ink accumulation								
Histamine 20 μg	R.P. 2786	—	±	±	—	—	—	—
	R.P. 6847	—	±	±	—	—	—	—
	Saline	++++	±	++	++++	++++	++++	++++
5-HT 0.2 μg	R.P. 2786	—	—	—	—	—	—	—
	R.P. 6847	++++	+	+	+	++	++	++++
	Saline	++++	—	+	+++	++++	++++	++++
48/80 0.2 μg	R.P. 2786	—	—	—	—	—	—	—
	R.P. 6847	++++	+	+	+	++	++	++++
	Saline	++++	+	++	+++	++++	+++	++++

Intensity of edema (per cent of maximum) as well as carbon accumulation in the skin where histamine, 5-HT, or 48/80 are injected I.D. Antihistamine (R.P. 2686 or R.P. 6847) are injected I.M. 25 min. before dextran. Stimulators I.D. and India ink I.V. are injected at regular time intervals after dextran. Each mark represents average readings on 5 rats.

outward flow of plasma fluids occur and when the accumulation of particulate matter in the capillaries throughout the entire body is inhibited by a mechanism still unknown. The storing of the particulate matter is by no means concomitant with the period of the hemoconcentration.

It is easy to assume a correlation between the alterations both in carbon removal and in endothelial activation since particles are retained in the blood during the same period in which there is no carbon accumulation in the capillary wall and no particulate matter can appear in the pericapillary space. It seems that a mechanism is paralyzed that mediates the acquired capacity of the capillary endothelium to accumulate particulate matter and to become permeable by particles at the site of an injury or stimulus when at the same

matter at the tubular surface of the capillary endothelium at the very sites at which later edema develops The particles soon penetrate and cross the capillary wall and as a sequel of the latter s increased permeability for particles appear in the pericapillary pace We suggest the term activation of capillary endothelium for this response In normal animals the same phenomena can be induced by stimuli or injury at any part of the body, in dextran treated rats however this can be done only before the appearance of edema

The reaction of endothelial activation is absent during the formation of dextran edema not only in the extremities but also in any other part of the skin During the period of edema formation only plasma fluid penetrate into the pericapillary ti sue from the vessels while particulate matter is re tained by the capillary wall

Simultaneously with the edema formation particulate matter is retained in the circulating blood If India ink is injected before the appearance or after the di appearance of edema the removal of carbon from the blood pro ceed at the same rate as in the normal rats These alterations in carbon removal during edema are not the consequences of hemoconcentration or of alterations in hemodynamics since there is evidence that they are not syn chronous in time The phenomenon of carbon congestion in the blood can be related rather to the effects of histamine and 5 HT As shown above the removal of the particles follows a normal course during the edema in the depleted rats

Activation of capillary endothelium and edema formation are two entirely different processes that are not observed concurrently Activation can also be separated from the edema in histamine and 5 HT depleted rats when the endothelial activation is absent whereas edema formation can be induced at full intensity Further experimental evidences in support of this double edged function of the capillary endothelium are supplied by the results obtained with the help of phenothiazine derivatives With specific inhibitors (R P 2786 and R P 6847) the endothelial activation is ab ent while the outward flow of plasma fluid results in edema formation This fact clearly shows that the two phenomena are governed by different mediators

It is obvious from these experiments that as injury or stimulus occurs the primary but distinct alterations in the function of capillary endothelium are the endothelial activation and edema formation These alterations can not be considered as capillary damage but as a purposeful and immediate defense response in case of necessity as a localization by the capillary endo thelium of the damaging agents their elimination into the ground substance of the pericapillary space and the aflux of plasma fluid into the damaged area Both the accumulation of particles and their elimination are processes of defense and neither occurs separately logic demands the common term of endothelial activation in order to distinguish this process of defense from the increased permeability for plasma fluids

How the particulate matter accumulates on the endothelial surface whether the phase of adsorption is due to an acquired stickiness of the cell and whether the particles are actively engulfed by the endothelial cells remain unanswered questions It is suggested by the experiments of Jancso[1] that when injury occurs the common endothelium is transformed into pecial endothelium

tamine 5 HT or both is normal although dextran edema develops at full intensity

Discussion

In the extremities of rats, intraperitoneally administered dextran induced early alterations in the function of capillary endothelial cells These alterations are characterized by accumulation of intravenously injected particulate

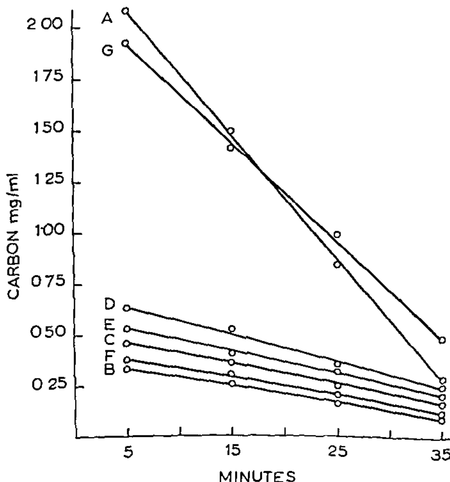


FIGURE 3 Removal of intravenously injected India ink from the blood of dextran treated rats India ink was injected 60 min after dextran when edema fully developed in all animals Carbon content in blood (mg /ml) is plotted against time in minutes after injection of India ink Key *A* normal rats received dextran *B* normal rats having received antihistamines (R P 3277 R P 4560 or R P 7044) and dextran *C* reserpine treated (5 HT depleted) rats having received dextran *D* polymyxin B treated (histamine depleted) rats having received dextran *E* 48/80 treated (histamine and 5 HT depleted) rats having received dextran *F* normal rats having received R P 2786 (inhibits the effect of histamine and the edema formation) *G* normal rats having received R P 6847 (inhibits only the effect of histamine) and 25 min later dextran

6 Heller J H R M Meier R. Zucker & G W Mast 1957 The effect of natural and synthetic estrogens on reticuloendothelial system function Endocrinology 61 735–741
7 West G B 1957 5-Hydroxytryptamine tissue mast cells and skin edema Intern Arch Allergy Appl Immunol 10 257–275
8 Alksne J F 1957 The passage of colloidal particles across the dermal capillary wall under the influence of histamine Quart J Exptl Physiol 44 51–66
9 Parratt J R & C B West 1957 5-Hydroxytryptamine and tissue mast cells J Physiol (London) 137 169 178
10 Parratt J R & G B West 1957 Release of 5 hydroxytryptamine and histamine from tissues of the rat J Physiol (London) 137 179 192
11 Gözsy B & L Kátó Investigations into the mechanism of action of neuroleptic drugs Indian J Med Research In press
12 Zweifach B W 1949 Basic mechanisms in peripheral vascular homeostasis factors regulating blood pressure 3rd Conf Josiah Macy Jr Foundation 13
13 Burke J F & A A Miles 1958 The sequence of vascular events in early infective inflammation J Pathol Bacteriol 76 1–19
14 Petry C J Csipák A Kovács & M Bentzik 1952 Neuere Beiträge zur Pathologie der pyogenen Entzündung II Die Wirkung des Antihistaminstoffes der Leukocyten auf den Verlauf der pyogenen Entzündung Arch intern pharmacodynamie 91 176–184

The activated endothelium in reality possesses the characteristics of the RES cells, namely adsorption and storage The importance of endothelial activation is also reflected in the experiments of Burke and Miles[13] and of Petry *et al*,[14] showing that the first 2 hours after the injection of microorganisms constitute the decisive period in the outcome of infections At this early phase the only tissue defense response occurs in the capillary wall In combatting the consequences of any injury, in eliminating damaging matter, and in the reparatory process the capillary endothelium plays a role certainly as important as that of the RES itself

Summary

Intraperitoneal injection of dextran in rats induced early changes in the function of capillary endothelium as manifested by the accumulation of intravenously injected carbon particles in the capillaries and, soon thereafter, in the pericapillary space of the face and extremities where edema develops later This capillary activation cannot be observed at the time when edema development is in progress and when simultaneously no India ink accumulation can be provoked by stimuli or injury at any other part of the capillary network During the progress of edema formation unusually high levels of carbon content were measured in the blood These changes are not the consequences of hemodynamic alterations since fall of blood pressure lasts but 10 min after dextran injection and the duration of hemoconcentration is not simultaneous with the time when India ink was retained in the circulation During the formation of edema plasma fluids but no particulate matter, appeared only in the pericapillary space After histamine and 5 HT depletion or after inhibiting the effects of liberated histamine with R P 6847 or inhibiting the carbon accumulation with R P 2786 edema was induced in full intensity with dextran Endothelial activation and edema formation are therefore two different and opposite processes No capillary damage was observed histologically after histamine dextran or 48 80 administration thus endothelial activation is considered a defense function rather than the result of capillary damage

Acknowledgment

We express our thanks to F Avon of Poulenc Ltd Montreal Canada for the phenothiazine derivates and to F J de Beer Wellcome Research Laboratories Tuckahoe N Y for the compound 48 80 used in these studies

References

1 HALPERN B N 1956 Histamine release by long chain molecules *In* Ciba Foundation Symposium on Histamine 92–128 Churchill London England
2 VOORHEES A B H J BAKER & E J PULASKI 1951 Reactions of albino rats to injections of dextran Proc Soc Exptl Biol Med 76 254–256
3 JANCSÓ M 1947 Histamine as a physiological activator of reticuloendothelial system Nature 160 227–228
4 BIOZZI G G MENE & Z ÓVÁRY 1951 Action of histamine and of leukotaxine on the permeability and granulopexy of the vascular endothelium Arch intern pharmacodynamie 86 335–349
5 MATOLTSY G & M MATOLTSY 1951 The action of histamine and antihistaminic substances on the endothelial cells of the small capillaries in the skin J Pharmacol Exptl Therap 102 237–249

The Reticuloendothelial System

Definitions

In the introduction I referred to the system as though it were a perfectly defined operational concept In the language of semantics the term reticulo endothelial system is a very high order abstraction Since in this paper the RES is the thing that is being stimulated by chemicals I must define what the RES means here In this paper it refers to those (heterogenous) cells scattered throughout living organisms in various stages of physiological growth metabolism and decay and subjected to a variety of localized environments of which phagocytosis is the common measurable characteristic

Delineation and Functions

Although in what follows phagocytosi will be used as the parameter by which to estimate stimulatory effects of chemicals there are many other known functions of RE cells While Policard [6] Halpern [7] and others have challenged Aschoff s conception of the functional unity of the RES we eek to avoid this issue by arbitrarily studying a function (phagocytosis) rather than a system However no cells exist that perform only the function of phagocytosis These same phagocytic cells are respiring metabolizing sub trates growing and cat alyzing anabolic and catabolic reactions many of which appear to be of the utmost importance to the survival of the total organism Some of these func tions include a role in the formation of antithrombins of fibrinogen and perhaps of antibodies [8,10] in detoxification of large molecules and aggregates [11,12] in the killing and digestion of bacteria [13,14] and in the breakdown of red blood cell fragments [15] It is quite improbable that the phagocytic index is an infallible key to what is happening to some of these other reactions that take place simultaneously in the same cells When one speaks of stimulation of the RES one must certainly specify carefully the biological level (organism organ or cells) and the cellular or intracellular reaction that one is considering since the multiplicity of reactions in a multiplicity of cells located in diverse physio logical environments practically ensures nonparallelism in their responses to external stimuli such as chemical compounds

Excellent over all delineation of the reticuloendothelial system exi ts [16] FIGURE 1 shows schematically the ramifications of the system as a whole Note that the process of phagocytosis does not appear although it is involved in each of the reactions shown thus it might be considered a fairly basic reaction At the risk of oversimplifying FIGURE 2 is included to indicate a working concept of this system In the funnel portion of this diagram where particles and solubles lie side by side arrows should indicate the interchangeability of these 2 states of aggregation Many examples are known of drugs that are injected as solubles but that finally become particles If the role of phagocytic cells is reflected at all accurately by this diagram the importance of detailed study of every aspect of the metabolism of these cells is certainly obvious since they involve decisions as to nutrition and disease sequelae and serve as partial determinants of normal physiological function

Admirable reviews of the role and function of the RES in relation to such

THE RETICULOENDOTHELIAL SYSTEM I CHEMICAL METHODS OF STIMULATION OF THE RETICULOENDOTHELIAL SYSTEM*

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INTRODUCTION

My assigned topic "Chemical Methods of Stimulation of the Reticuloendothelial System" (RES) seems rather too inclusive since chemical intermediaries are released by almost every type of stimulus. If one were to discuss literally the chemical methods of stimulation of the RES one would monopolize this entire monograph since in the final analysis most influences on the RES at a cellular and physiological level are chemical.

I shall therefore limit this paper to a discussion of the influence of some exogenously supplied chemical compound upon the system. In so doing it is not assumed that I am dealing with uncomplicated effects since (1) the administration of any chemical or drug by a parenteral or oral route involves a variable amount of concomitantly administered stress to the animal and (2) the detailed mode of action of the chemical or drug on the RES *in vivo* therefore involves in addition to the effects produced by the drug itself responses to physical trauma involved in the procedure of administration. These effects may be minimized, but they cannot be eliminated. The best that one can hope to do is to make them as uniform as possible throughout the experimental groups.

The point to emphasize is that we are always dealing with the effects produced by a combination of physiological stimuli on the RES. The specific problem in studying the chemical stimulation of the RES is so to standardize the procedures and the biological material as to be able to assign any changes we observe in function to the biochemical interactions of the compounds being investigated rather than to background effects produced by the technique; such effects must be kept minimal. Hence the multiplicity of the variables and the complexity of the problem of estimating the chemical stimulation of the RES is immediately apparent to the investigator entering this field even prior to his selection of any given test or battery of tests, for background influences will affect drastically any assay chosen. Basically we are dealing with a system so sensitive to external stimuli of all types as to challenge the very best efforts of the investigator in devising suitable tests with suitable controls for the analysis of its normal function.

Such diverse environmental influences as starvation[1], X irradiation[2,3], environmentally conditioned and autonomic nervous reflexes[4] and genetics[5] affect the ability of the system to react to hormones, drugs and chemicals.

* This paper is the first of a series of papers emanating from this department on the effect of various groups of drugs on the reticuloendothelial system.

things as immunological processes[7] *speicherung**[17] mast cells[15] swimming stress[19] and cancer[6] exist Detailed information of the finer processes of metabolism of these cells is lacking however Between the chemical or drug and its metabolism lie the processes of phagocytosis and the influence of drugs and chemicals on phagocytosis which I now propose to consider

Level of Organism

A sample of the way in which phagocytic cells are distributed in organisms is shown in FIGURE 3 In most studies of phagocytosis the liver shows preponderant localization although this localization is not necessarily proportional to the total number of phagocytic cells present Other factors such as proximity to blood supply quantity of blood supply and the local nutritional environment of the cell condition the relative proportion of uptake A general picture similar to this has been obtained in many different animals with many different colloids and with thorium[1] and chromium phosphate[2?]

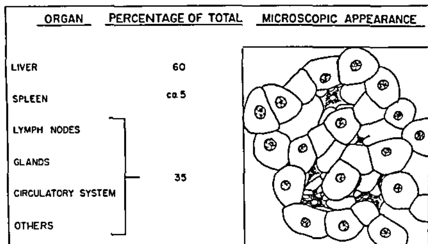


FIGURE 3 Schematic diagram of the location and appearance of RE cells

Level of Organ

The distribution of colloidal preparations in various organs has been studied extensively for example in liver[24] spleen[5] lymph nodes capillaries and endocrines Distribution in liver has been of major interest The tendency toward concentric localization around the central vein has been commented upon many times[26] It has been verified that the cells that remove colloidal particles in the liver are the Kupffer's cells and that they remove as much as 95 per cent of the total injected material depending upon its particle size distribution and the quantity of material injected[27] The reaction is extremely rapid the material being removed exponentially in such a fashion that if small

* The reversible dynamic process of concentration and storage of metabolites or drugs

FIGURE 1 Some postulated intermediary relationships of the RES (Diagram by E. R. Gabrieli.)

FIGURE 2 Schematic diagram of the functions of RE cells

blood flow, and geometry of cells with respect to capillaries or arterioles All of the factors that bear on cell growth and maintenance *in vivo* must be considered variation of these factors from site to site in a complex organ may produce phagocytic indices on a cellular or organ basis which vary over a large range (with respect to rate)

Level of Intracellular Biochemical Function

At this level of organization we are concerned with what occurs in the various metabolic compartments of the cell 'fermentation vessel (FIGURE 5) How does the macrophage differ in its metabolic machinery (enzymes and architecture thereof) from nonphagocytic cells? Is phagocytosis related to Q_{O_2}, to proteinase activity or to phosphatase activity and what are the underlying

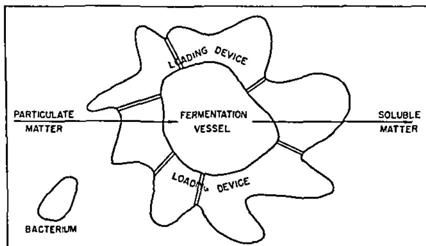


FIGURE 5 Schematic diagram of an RE cell illustrating the function of its various parts

energetic reactions of phagocytosis that are affected by a chemical or drug that modifies the phagocytic rate? What is the relationship of eosinophils and phagocytic cells to RES and antibody formation?[37, 38] What reactions must stimulate or inhibit in order to affect processes such as non pecific resistance antibody formation allergic phenomena and other conditions that are thought to be correlated with macrophage chemistry?

Concepts of Stimulation with Respect to These Different Levels of Organization and Heterogeneity of Cell Type

If we admit that healthy functioning phagocytic cells are important to the organism as a whole the purpose for chemotherapy of the RES should be to produce and maintain normal healthy function of the RES as it exists in young healthy animals This may involve either stimulation or depression of any of the known functions of RES One of the things that is done in immunization

quantities of colloid are used the technique measures rate of liver blood flow[9] For measuring the rate of phagocytosis specifically (phagocytic indices) *in vivo* the conditions outlined by Benacerraf *et al* [5] are convenient There seems to be some indecision as to the type of colloid that is most satisfactory in practice carbon chromium phosphate and iodinated denatured albumin all having been used with significant results These materials can be used to estimate over all phagocytic function in an organism an organ (by perfusion techniques) or in isolated cells (monocytes for example) I discuss the technique below under the heading *Effects of Specific Drugs*

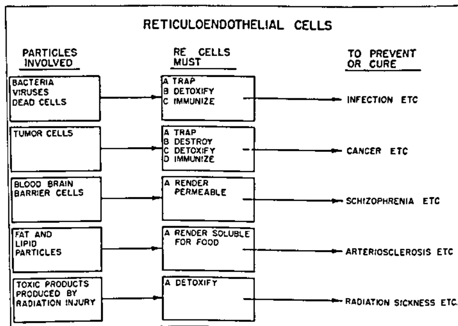


FIGURE 4 Schematic diagram of the possible implications of the functions of RE cells in the treatment of various disease states

Level of Cells

One might consider RE cells as chemical factories consisting of (1) devices for collecting appropriate raw materials (2) fermentation vessels equipped with inocula (enzymes) to perform many functions and (3) a product distribution system to distribute end products to the circulatory system and hence to other body cells (FIGURE 5)

FIGURE 4 indicates the possible implications these processes have for the treatment of various disease states This paper focu es on the trapping mechanism and the efficiency of these cell in o far a it i influenced by exogenous chemicals and drugs

In the immediate environment of a cell many factors may influence phagocytosis medium [9] mobility [30, 31] competitive effects by other colloids[27] rate of

sient and extremely variable over the course of an infection Hence studies attempting correlation must follow phagocytic function in detail a requirement necessitating serial measurement of the function in the same animals that receive the infective challenge If such requirements are not met the problem of proving one to-one correspondence between elevated phagocytic rate and elevated resistance to an actual infection becomes a complicated statistical problem in which suitable control groups are lacking It is in such method that the use of inbred strains of animal is essential in order to minimize the otherwise insurmountable genetic variation in individual resistance [4] Similar principles apply to the use of phagocytosis *in vivo* as a parameter for estimation of degree of stimulation produced by various drugs or chemicals [68]

Inversion of effects of chemical stimulation as a function of dose and test time In previous investigations of chemical stimulation of phagocytosis there is another fact of importance usually investigated inadequately This is the extension of the old and well known principle that for most poisons or drugs a curve of a given dependent variable often follows a bimodal shape stimulation at low levels and inhibition at high levels [70] This observation is especially apparent in enzyme kinetics where even extremely toxic poisons produce marked stimulation at very low concentrations followed by inhibition at high concentrations Something similar to this occurs with many drugs when they are tested over a wide range of concentrations using *in vivo* phagocytosis as the measured parameter This phenomenon is illustrated in detail below where corti one is discussed

These general remarks on reticuloendothelial function and phagocytosis are by way of offering introduction to and some reservations concerning the discussion of specific studies that follows

Effects of Specific Drugs

Antibiotics No attempt is made here at exhaustive coverage A review of the effects of antibiotics on phagocytic function is in preparation [71] It is proposed here to discuss briefly some a pects of the work that has been done with the tetracyclines as illustration of the potentialities of interpretation in this field

Early distribution studies using ultraviolet microscopic fluorescence[72] indicated that oxytetracycline seemed to distribute itself preferentially in the lymphoid tissue spleen and Kupffer s cells of the liver This observation has been confirmed in detail with radioactive tetracycline[73] and with radioactive oxytetracycline [74] That this localization might be followed by special effects on phagocytic properties of the cells was an interesting speculation [74] Early studies indicated no profound effect of penicillin tetracycline oxytetracycline or oleandomycin on the relative phagocytic velocity of inbred mice 2 and 24 hours following a single do e of 10 mg /kg I P [71] Although there is no apparent effect under these conditions oxytetracycline has been found to have an influence on the stimulation in phagocytosis produced by lipopolysaccharides (LPS) [71]

Under certain conditions oxytetracycline reduces the relative rate of phagocytosis when administered concurrently with LPS The differences are small

involves the conditioning of this system [34] There are perhaps many procedures of modern medicine that act in a similar manner This possibility should become clearer as further work is reported on the relation of stimulation of phagocytic cells to pathological states

Relation of Stimulation of Phagocytosis to General Physical Reactions of Interest to Medicine

Infection has been implicated many times as a sequent to lowered RE function [35,36]

Important moderation of RE function is probable in hemorrhagic shock [37] immunization,[38] physicochemical alteration and homeostasis [39] cholesterol imbalance [40] thermal injury [41] multiple sclerosis [42] rheumatoid arthritis,[43] hypothermia [44] infectious mononucleosis [45] atherosclerosis [46] dextran storage [47] cancer [48] and tuberculosis [49] these conditions have their echoes if not their determinants in the RES

The problem of this series of papers, of which this is the first is to try to review the way various drugs having more or less well known primary uses in medicine (vitamins antibiotics corticosteroids tranquilizers antihistamines, and various macromolecules such as lipopolysaccharides heparin, and polyvinyl pyrrolidone) affect the function of phagocytosis when it is measured under controlled conditions Conversely the influence of some of these compounds on phagocytosis may throw light upon some of the side effects or give clues to possible unrealized bioactivities of these drugs

Brief review of techniques The physiology of phagocytosis of particles has been reviewed by Benacerraf *et al* [50] likewise the physiological stimulation and inhibition of the phagocytic function of the RES have been considered by Heller[51] and others The following pertinent studies bearing on function have been considered sensitivity of phagocytic cells of liver to alterations in plasma proteins [52] the effects of alpha radiation on function [53] normal and pathological values for radioactive gold clearance [54] the kinetics of the phagocytic process [55] differential effects produced by particle size [56] dependence of process of phagocytosis on carbohydrate metabolism [57] factors controlling the process[58] and artifacts in the determination of phagocytosis [59] phagocytosis promotion factors [60] relationship of micellophagosis and detoxification to phagocytosis [11] use of phagocytic functions in investigating blood flow [61] estimation by means of C^{14} and I^{131} labeled plasma proteins [62] effects of blockade of phagocytosis [22] use of prodigiosin for estimation [63] function tests in human patients [64] dye tests with colloidal suspensions of specific diameter [65] use of heat denatured human serum albumin tagged with I^{131} [66] effect of size distribution on speed and distribution in body [24] and function of phagocytosis in fat metabolism [67]

However the problem of setting up a routine screening technique amenable to estimation of chemical stimulation and comparisons over long periods of time has been given relatively slight attention [68]

Correlations of phagocytic rate with resistance The degree to which phagocytosis per se has been related to resistance to infection and other forms of graded trauma is slight A broad study of this correlation was reported by Biozzi *et al* [69] It is well to remember that phagocytic function may be tran

shown to have significant moderating properties on phagocytosis However the variations of the e effects with concentration have not been elucidated in the literature

Cortisone Corti one was reported to block phagocytosi by the RES and facts are well known that substantiate the thesis that corti one will reduce the re istance of animal to many kinds of infection [110 115] However the contrary

TABLE 1

CrPO₄ T ½ CONTROL VALUES OVER A 2 MONTH PERIOD

S ss wh t m l	C57BL m l †	Stra A m l †
0 78	0 63	0 80
0 60	0 69	0 94
1 45	0 63	0 82
1 23	0 69	0 60
0 81	0 67	1 23
1 32	0 70	0 82
1 16	0 72	0 58
1 03	0 74	0 60
0 94	1 14	0 84
0 61	0 77	0 85
0 77	0 72	0 88
0 88	1 05	0 60
0 48	0 85	0 77
0 83	1 14	0 73
0 60	0 85	0 67
1 08	0 83	1 10
0 74	0 91	0 58
0 7'	0 98	0 59
1 95	0 90	0 69
1 55	0 91	0 80
0 73	0 86	0 81
0 55	0 92	0 75
0 74	0 86	0 65
1 94	0 55	0 66
0 54	0 77	0 71
0 88	0 ,0	0 93
4 27	0 76	0 70
0 45	0 88	0 69
1 06 ± 0 745	0 87 ± 0 148	0 76 ± 0 156

Commercially supplied
† Bred and raised in our own laboratory

effects have also been observed for example cortisone under certain conditions will enhance the resistance of animal [116 1 0]

In an effort to help explain such bimodal action a study of the influence of cortisone and other steroids on phagocytosis was undertaken The precise relationship between chromium phosphate removal time and resistance to infection in cortisone and hydrocortisone treated inbred animals has not been elucidated

Chromium phosphate technique The chromium pho phate technique employed here has been described elsewhere [69] FIGURE 6 illustrates the usual homogeneity encountered in a group of 5 animals when the test is performed on

but significant and reproducible In conjunction with data showing that LPS is also localized by phagocytic cells[75] the possibilities of mutual interference or mutual synergism are apparent Further examples of this will be discussed in the section on corticosteroids Detailed studies of the interaction of antibiotics and LPS are forthcoming[71] Considerable data in the literature have been amassed regarding the influence of antibiotics on phagocytosis or other parameters of the RES[78 90] The variety of techniques used makes correlation of these data in tabular form difficult However the interest in this field is on the increase for the sufficient reason that in general the anti

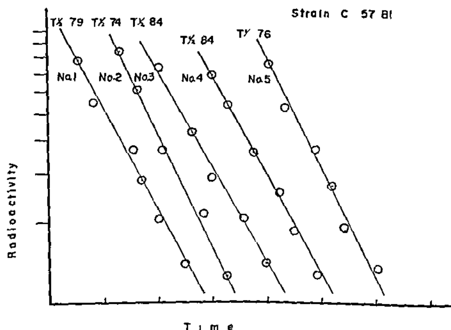


FIGURE 6 Results of the CrPO₄ technique on 5 mice Note the usual homogeneity of results when the test is performed within a single day with inbred strains

biotics have been found to have important effects either positive or negative on phagocytosis and the related processes of immunity

Vitamins Effects of vitamins on phagocytosis are difficult to disentangle from their general effects on nutrition However immediate stimulatory effects have been indicated for ascorbic acid (in mice)[91] choline[9?] vitamin B_{12}[93] and others Again the lack of uniform criteria for estimating degree of stimulation makes intercomparisons difficult In most cases the stimulation produced seems to be transient lasting less than 24 hours

Other classes of drugs The antihistamines[94] some ataractic agents[95 96] LPS[75] and other macromolecules such as heparin[97 98] azulene compound foreign body particles and other poorly defined material[9] phagocytin[10] malucidin[101 10] piromen[98] properdyl[103] various toxin[?] phlorizin[106] novocaine[107] polyvinyl pyrrolidone[108] mucin[17 69] and lipids[109] have all been

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0 61	0 77	0 85
0 77	0 72	0 88
0 88	1 05	0 60
0 48	0 85	0 77
0 83	1 14	0 73
0 60	0 85	0 67
1 08	0 83	1 10
0 74	0 91	0 58
0 72	0 98	0 59
1 95	0 90	0 69
1 55	0 91	0 80
0 73	0 86	0 81
0 55	0 97	0 75
0 74	0 86	0 65
1 94	0 55	0 66
0 54	0 77	0 71
0 88	0 70	0 93
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a single day with inbred strains. The variation encountered in control values from day to day is reflected in the data of TABLE 1.

Importance of uniform animals for phagocytic tests. No study has reported comparative data on the extent of daily variation in average phagocytic rates within various types of animal colonies. During the past 5 years we have studied this inherent and conditioned variability. We have compared randomly bred commercial wild strain with inbred (from brother-sister mating) animals produced in our own laboratory (TABLE 1). Since large variations were encountered in commercial Swiss white males, even when animals of uniform sex and age were tested, it was determined early to deal only with carefully standardized hybrid and pure inbred animals produced within our own laboratory from brother-sister matings. The groups were selected with respect to uniform sex and weight and preconditioned, for at least 2 weeks to

TABLE 2

THE EFFECT OF VARIOUS STEROIDS AT CONSTANT DOSE* ON RELATIVE RATES OF CHROMIUM PHOSPHATE REMOVAL IN STRAIN A MALES

Compound	Relative removal rate†
Suspending agent‡	100
11-Deoxycorticosterone	100
Corticosterone	145
Etiocholanolone	110
3 α-Hydroxypregnane-20-one	95
Reichstein's compound S	80
Hydrocortisone	190
Cortisone	135

* Intraperitoneal administration 0.5 mg./kg.; chromium phosphate injected 24 hours later. Each number represents 10 animals.

† Untreated normal controls arbitrarily set at 100. Relative removal rate

$$= \frac{T\ 1/2 \text{ control group}}{T\ 1/2 \text{ test group}} \times 100$$

‡ Pfizer suspending agent: 0.9 per cent benzyl alcohol, 0.9 per cent NaCl, 0.4 per cent Tween 80, 0.5 per cent Na carboxymethyl cellulose.

the testing laboratory prior to test, to obviate the known large effects of stress on the circulation and phagocytic mechanism being measured.[5] The testing laboratory is an air-conditioned, temperature- and humidity-controlled animal room. Under such conditions standard deviation of phagocytic rates within a group of 5 animals were routinely of the order of 5 to 15 per cent. When special care was needed in assaying small effects, the method of serial determination on randomized groups of littermates in which each animal could be used as its own control was used.[53]

Experimental results. In studying the activity of these steroids, several questions were of special interest: (1) How specific were the stimulatory or inhibitory effects encountered? Was the chromium phosphate procedure used subject to large numbers of false positives (produced perhaps by colloidal structure or dispersion)? Would the technique respond to specific chemical structures? Also, what are the conditions (such as dosage range and route) under which stimulation may be produced?

With regard to all but the last of these questions, TABLE 2 presents data suggesting that the test has rather marked specificity. Three compounds produced a rather prolonged stimulation of the removal rate (>24 hours) of corticosterone, hydrocortisone and cortisone. A group of 33 other steroids tested in a similar program gave essentially negative results. The effects produced by these 3 compounds are considered to be highly significant, although the exact mechanism by which they were produced is not clear.

TABLE 3
THE EFFECTS OF VARIOUS LEVELS OF COMPOUND S AND CORTISONE ON RELATIVE REMOVAL RATES IN C57BL MALES

Compound	Level mg/kg	Relative removal rate
Suspending agent	—	100 ± 3
Compound S	0.5 5.0 50.0	69 ± 6.4 55 ± 20 145 ± 13.4
Cortisone	0.5 5.0 50.0	145 ± 16.0 143 ± 9.7 154 ± 7.2

Indicated dose given twice at 24 hour intervals intraperitoneally to 20-gm. mice in 0.4 ml. suspending agent. 24 hours following the last dose, chromium phosphate half times were measured.

TABLE 4
THE EFFECTS OF CONSTANT DOSAGE OF CORTISONE, HYDROCORTISONE AND REICHSTEIN'S COMPOUND S ON CHROMIUM PHOSPHATE REMOVAL

Compound	Route	Relative removal rate
Suspending agent	I.P.	100 ± 5.0
Cortisone	I.P.	110
Dihydrocortisone	I.P.	170
Compound S	I.P.	64
Suspending agent	I.V.	100 ± 10.0
Cortisone	I.V.	210
Hydrocortisone	I.V.	186

Measured 1 hour following administration of 5 mg./kg. to 20 gm. C57BL mice.

Although these effects were more difficult to demonstrate on commercially supplied *wild strains* because of the inherent variations in removal rates, the effect was first picked up in commercial Webster Swiss albino mice and has been repeated since in strain A and in the hybrid strain LAF LABF. The effect is quantitatively less but still present in strain C57BL. However, because of the greater uniformity of strain C57BL (TABLE 1) the effect was studied further in this strain.

Since a great deal is known about the relative bioactivities of Reichstein's compound S and cortisone free alcohol,[11] their relative activities in this test were a point of interest (TABLE 3). As with strain A males, the first effect of

compound S was an inhibition of removal rate, however at very high concentrations some stimulatory effect was obtained. Similarly, compound S demonstrated no antiphlogistic potency until a large amount of it was present.[121] Cortisone free alcohol, on the other hand, produced stimulation at all levels in these experiments, but the stimulation did not prove to be proportional to dose within the range of 0.5 to 50.0 mg./kg.

If activity such as that displayed in TABLE 3 occurred, the compound was

TABLE 5

EFFECTS OF 4 COMPOUNDS ON RELATIVE REMOVAL RATES IN C57BL MICE 1 HOUR FOLLOWING INTRAVENOUS ADMINISTRATION

Compound	Dose mg./kg.	
	0.5	5.0
Suspending agent	100 ± 10	100 ± 10
Compound S	108 ± 10	105 ± 20
Hydrocortisone	113 ± 20	222 ± 10
Cortisone	105 ± 20	167 ± 20
Progesterone	80 ± 50	75 ± 60

TABLE 6

EFFECT OF COMBINATIONS OF LIPOPOLYSACCHARIDE AND CORTISONE ACETATE ON RATE OF REMOVAL OF CHROMIUM PHOSPHATE

Compound	Relative removal rate	
	Experiment I	Experiment II
Suspending agent	100	100
Hydrocortisone	90	95
Cortisone	85	90
Cortisone acetate	85	50
Cortisone acetate + LPS	220	120
LPS	190	115

All compounds were administered intraperitoneally 10 mg./kg. Lipopolysaccharide (LPS) was administered with the cortisone acetate at a level of 1.0 mg./kg. Chromium phosphate half times measured 24 hours later.

investigated for activity at shorter periods of time, although such measurements are less specific due to stress effects on the test. TABLE 4 summarizes the data obtained when chromium phosphate removal was measured 1 hour following intraperitoneal and intravenous injection of the steroid.

TABLE 4 shows that the stimulation found at 24 hours occurred also as early as 1 hour and could be produced by intravenous as well as intraperitoneal administration. Furthermore, blockade by compound S also was apparent at 1 hour. A new feature was the increased activity of cortisone when given by the intravenous route. Whereas it had almost no activity relative to dihydrocortisone at 1 hour intraperitoneally, it showed fully comparable activity in

travenously When a dose of 50 mg/kg was administered intravenously severe blockade occurred

The differences in the specific activity of cortisone and hydrocortisone when given by the intravenous route are indicated in TABLE 5

Cortisone produced a greater effect on the 1 hour half times at a significantly lower dosage than did hydrocortisone Progesterone had no stimulatory effect it produced if anything an inhibition but the standard deviations of these groups were huge compared to those administered steroid compound Apparently there was a great deal of individual variation in the response of these animals to progesterone under these conditions

The decrease in effect at 5 0 mg/kg over the effect at 0 5 mg/kg showed the presence of an overriding second factor which at levels higher than 5 0 mg/kg resulted in inhibition of relative removal rates This effect seemed to achieve a plateau around 10 mg/kg for a single dose regimen It is interpreted as due to a blockade of the Kupffer s cells that are mainly responsible for the chromium phosphate removal

LPS materials can overcome the blocking effect of high doses of cortisone acetate (TABLE 6) They produced stimulation of removal rates in the presence of both the mild and marked inhibition produced by high levels of cortisone acetate

Discussion

A discussion of the chromium phosphate test as used for these studies has been published elsewhere [63] and useful discussions of the factors influencing phagocytosis of colloidal particles are in the literature [28, 177]

The mechanism of stimulation or inhibition of the rate of removal of chromic phosphate from the blood by cortisone and hydrocortisone is not clear for the following five reasons

(1) A material that coats either the phagocytic cell or the colloidal particle may affect the rate of removal by altering the charge the interfacial tension or specific chemical affinities

(2) A material that stimulates the metabolism involved in producing energy for phagocytosis may stimulate phagocytosis one that inhibits these processes may inhibit it

(3) A material that alters either the rate of blood flow or the geometry and time of contact between colloid and phagocytic cells may affect the phagocytic rates

(4) A material that furnishes opsonic factors for phagocytosis may stimulate the process

(5) A material that increases the ability of phagocytic cells to digest phagocytized material translocates them to a more favorable position or one that stimulates the production of new phagocytic cells from precursor cells may produce stimulatory effects

No attempt is made or inferred here to relate the parameter of relative removal rates directly and solely to phagocytosis because of the multiplicity of possible mechanisms suggested above The susceptibility of the process to circulation time was not considered to be an important drawback in these

studies Circulation time and circulation dynamics are extremely important factors in determining how rapidly a material becomes phagocytized and probably should be included in *in vivo* attempts to relate phagocytic rate to resistance Thus any marked effect of a compound on circulation dynamics through the liver or in circulation times would most likely be very pertinent to the rapidity with which invading bacteria were sequestered by the elements of the RES

In considering the specificity of the test the fact that there are very few compounds that will alter the removal rate significantly and retain an effect after 24 hours duration (*see* TABLE 2 and its context) is very significant Thus it would appear that the frequency of compounds possessing the ability to produce either prolonged changes in circulation dynamics or changes in phagocytic dynamics was comparatively low in the series of steroids tested

It may be stated in passing that the number of positive or negative indications from the chromium phosphate phagocytosis test increased as the testing time approached closer to the injection of the test compounds This phenomenon is especially true in the period from 5 min to 1 hour It is probably due to a composite of effects such as stress due to handling and short term activities on both circulatory and phagocytic systems that do not persist The 24 hour test employed however does give a rapid and practical method of indicating antagonisms or synergisms between drugs (for example LPS and cortisone) and of evaluating roughly the biological intensity of the total effect

It has been shown by others[1] that high doses of cortisone given to Swiss albino mice produced marked inhibition of phagocytic velocity The effect was very slight at 25 mg/kg, but very marked at 250 mg/kg In C57BL mice the latter dose produced marked inhibition immediately and 1 hour following administration but the rate had returned substantially to normal at 24 hours Hence in studies attempting to correlate phagocytic inhibition with increased susceptibility to infection it is of the utmost importance that one follow phagocytic rates continuously prior to and following administration of the infecting dose especially when dealing with high doses of cortisone where the initial effect is blocked but the later effect may be stimulation of phagocytic velocity For these reasons correlation of removal rates with resistance to infection will require repeated assays of removal rates The capacity of a compound to produce or maintain rapid phagocytosis throughout an infection is possibly related more closely to its effect on survival than its ability to produce a transient stimulation in phagocytic rate prior to infection as measured by a single determination of removal rate Thus mucin administration to mice partially blocked phagocytosis coincidentally with this a decrease in resistance to challenge by *Staphylococcus aureus* existed[68] this is the famous and well known ability of mucin to enhance virulence[13] If the phagocytic rate was measured 12 hours following mucin administration however an increased rate was found subsequent challenge of the animals now indicated an increase in resistance[14]

As previously pointed out many compounds other than cortisone and hydrocortisone have been proved to produce both inhibitory and stimulatory effects (not necessarily in that order) as a complex function of the dose and of

time of measurement following administration With cortisone both effects (blockade and stimulation) were seen within a 24 hour period following intravenous injection of the proper (large) dose both effects were produced with I I S but as much as 2 weeks was required to trace them fully following a single dose (Snell and McBride unpublished data) To follow such changes the chromium phosphate test utilizing small amounts of colloid was considered to interfere least with the phagocytic system under conditions of repeated measurements in the same group of animals[65]

From the standpoint of prophylactic or therapeutic use of corticosteroid it may be well to guard against oversimplifying the situation Low levels of cortisone and hydrocortisone may produce beneficial stimulation immediately following administration It is well to remember however that individual differences as to what would constitute a low dose of cortisone make this fact very difficult to apply in a practical situation If human responded as uniformly to these compound as do strains of brother sister mated mice the application might be possible

For the same reason it is difficult to assess what constitutes a blocking dose in an individual case It is possible that a blocking dose may vary by 1 or 2 orders of magnitude depending upon the state of health of the individual The fact that cortisone blocks phagocytosis at high levels immediately or several hours after administration must not be interpreted in a prophylactic situation to mean that this effect will predominate no matter when the animal receives an infective challenge In a therapeutic situation it does not mean that the presumably unfavorable effects of a temporary blockade will necessarily predominate over the presumably beneficial effects of the subsequent stimulation It would seem that everything may depend upon a complex function of the relative virulence of the infection and the time required for stimulation to succeed the blockade Of greatest importance perhaps is the period of time over which stimulation may be maintained

These speculations of course have excluded consideration of the various other functions of the RES such as the general health of the macrophages with respect to their ability to kill the phagocytized material and the degree of phagocytic translocation of virulent organisms that may result in other foci of infection In limiting the scope of these speculations to the possible relationship between removal rates and infection we merely recognize the fact pointed out by others[6] that the parameters involved in the phagocytic rate are at the present time easier to study *in vivo* than are those of phagocytic metabolism and translocation

Summary

The necessity has been stressed of defining the RFS carefully in terms of biological level (animal organ cell or intracellular processes) and function (such as phagocytosis and metabolism) in order to discuss stimulation of the RES in an organized fashion

The possible implications of stimulation in terms of its relationship to desirable clinical ends have been described

Studies of stimulatory effects on phagocytosis produced by low concentrations of certain corticosteroids led to the five following conclusions

(1) Cortisone and hydrocortisone at levels below 5 mg/kg produce a marked stimulation in clearance rate of chromium phosphate from the blood of C57BL, strain A and LAF LABF mice The effect persisted longer than 24 hours

(2) The effect seemed to be relatively specific since compound S progesterone deoxycorticosterone etiocholanolone, 3-α hydroxypregnane 20-one and 33 other steroids screened showed no such effect Corticosterone produced the effect to a smaller extent Compound S produced some stimulation at very high levels (TABLE 2)

(3) All of these compounds at higher doses (250 mg/kg) produced blockade"

(4) Speculations were made concerning the implications of stimulation and depression of relative removal rates in infectious processes it is suggested that the bimodal action of cortisone and hydrocortisone on phagocytosis as a function of dose level may help to explain conflicting observations as to the deleterious or beneficial action of cortisone in disease processes

(5) It is still too early to discern a unifying principle or correlation between the rate of phagocytosis and the various activities of large groups of drugs however some apparent relationships can be postulated in the case of certain individual drugs such as cortisone

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References

1 Di Luzio N R & B S Powers 1956 The effect of starvation on the phagocytic activity of the reticuloendothelial system RES Bull II(2) 25

2 Gabrieli E R & A A Auskaps 1953 The effect of whole body X irradiation on the reticulo-endothelial system as demonstrated by the use of radioactive chromium phosphate Yale J Biol Med 26 159

3 Esplin D W S Marcus & D M Donaldson 1953 Effects of X irradiation and adrenalectomy on phagocytic activity J Immunol 70 454

4 Gordienko A N 1957 A few words regarding the reflex agency in immunogenesis A continuation of the discussion on The reflex agency in the mechanism of antibody production J Microb Epidemiol Immunol 28 138

5 Gardner W U 1958 Genetic aspects of hormonal influences in cancer Ann N Y Acad Sci 71(6) 1097

6 Policard A 1957 The morphology and physiology of the reticulo-histiocytic cell *In* Physiopathology of the Reticulo-Endothelial System B N Halpern Ed 12 Blackwell Scientific Publ Oxford England

7 Halpern B N 1959 The role and function of the reticulo endothelial system in immunological processes J Pharm und Pharmacol 11(6 321

8 Slatis P 1958 The synthesis of prothrombin and proconvertin in the reticulo endothelial system an experimental study on rats Scand J Clin Lab Invest 10 (Suppl 33) 73

9 Roberts K B 1955 The failure of macrophages to produce antibodies Br J Exptl Pathol 36 199

10 Speirs R S 19 8 A theory of antibody formation involving eosinophils and reticuloendothelial cells Nature 181 681

11 Higginbotham, R. D & T F Dougherty 1956 Micellophagosis and detoxication RES Bull II(2) 27

12 HIGGINBOTHAM R D & T F DOUGHERTY 1957 Micellophagosis RES Bull III(2) 19
13 ROWLEY D & A C WARDLAW 1958 Lysis of Gram negative bacteria J Gen Microbiol. 18(2) 529
14 ROWLEY D 1958 Bactericidal activity of macrophages *in vitro* against *E coli* Nature 181 1738
15 MIESCHER P 1957 The role of the reticulo-endothelial system in haematoclasia *In* Physiopathology of the Reticulo-Endothelial System 155 B N Halpern Ed Blackwell Scientific Publ Oxford England
16 BAILLIF R N 1956 Delineation of the RES RES Bull II(1) 3
17 JANCSÓ N 1955 Speicherung Stoffanreicherung im Retikuloendothel und in der Niere Akademiai Kiádó Budapest Hungary
18 RILEY J F 1957 Functions of the tissue mast cells RES Bull III(2) 18
19 BRODIE B B 1956 Pathways of drug metabolism J Pharm and Pharmacol 8 1
20 STERN K 1941 Investigations on the reticulo-endothelial function of cancer patients J Lab Clin Med 26 809
21 ENGELS V W MAURER & A NIKLAS 1949 Thorium resorption in the reticulo-endothelial system Z ges exptl Med 115 221
22 GABRIELI E R & H HOLMGREN 1952 Studies in the blockade of the reticulo-endothelial system Acta Pathol Microbiol Scand 31 205
23 MCCORMICK J B G MILLES B JAFFE & I SEED 1954 Tissue distribution of injected radioactive colloidal chromic phosphate A M A Arch Pathol 58 187
24 JALLUT O L PEGUIRON P LERCH S NEUKOMM & R FEISSLY 1955 Speed of disappearance from the blood and distribution in different organs of radioactive colloidal CrPO as a function of the injected particle size RES Bull 1(3) 70
25 GYI K K & S MARCUS 1957 Localization of i v injected thorium dioxide in the spleens of normal and X radiated mice A measure of phagocytic activity RES Bull III(1) 7
26 GABRIELI E R 1952 The distribution of radioactive chromium phosphate in tissues of rodents following intravenous injection Acta Pathol Microbiol Scand. 31 195
27 BIOZZI G B BENACERRAF B N HALPERN & C STIFFEL 1957 The competitive effect of certain colloids on the phagocytosis of other colloids by the cells of the reticulo-endothelial system and the phenomenon of phagocytic preference RES Bull III(1) 3
28 BENACERRAF B G BIOZZI B N HALPERN & C STIFFEL 1957 Physiology of phagocytosis of particles by the R E S *In* Physiopathology of the Reticulo-Endothelial System 52 B N Halpern Ed Blackwell Scientific Publ Oxford England
29 RABSON A S F Y LEGALLAIS & S BARON 1958 Adaptation to serum free medium by a phagocytic cell strain derived from a murine lymphoma Nature 181 1343
30 MILES A A 1957 Local defences against infection and their relation to the general reticulo-endothelial defences *In* Physiopathology of the Reticulo-Endothelial System 188 B N Halpern Ed Blackwell Scientific Publ Oxford England
31 NICOL T & D L J BILBEY 1958 Elimination of macrophage cells of the reticulo endothelial system by way of the bronchial tree Nature 182 192
32 SPEIRS R S 1957 Relation of eosinophils to antibody formation RES Bull III(1) 19
33 COPE O & L ROSENFELD 1946 The lymphatic system Ann Rev Physiol 8 297
34 DONALDSON D M S MARCUS K K GYI & E H PERKINS 1956 The influence of immunization and total body X irradiation on intracellular digestion by peritoneal phagocytes J Immunol 76 192
35 DAVYDOVSKII I V 1956 Uchenive ol infektsii Medgiz U S S R
36 DAVIE A M 1952 The reticulo-endothelial system in resistance to bacterial disease Acta Med Orientalia Israel Med J 11 165
37 ZWEIFACH B W & B BENACERRAF 1958 Effect of hemorrhagic shock on the phagocytic function of Kupffer cells Circulation Research 6 83
38 GABRIELI E R 1955 Reticulo-endothelial activity during antibody response Ann N Y Acad Sci 59(3) 337
39 NEUKOMM S 1955 Physicochemical factors governing the phagocytic function of the RES RES Bull I 53
40 FRIEDMAN M & S O BYERS 1954 Observations concerning the production and excretion of cholesterol in mammals XIV The relationship of the hepatic reticulo-endothelial cell (Kupffer cell) to endogenously produced cholesterol Circulation 10 491

41 WARNER G I & I L DOBSON 1954 Disturbances in the reticulo-endothelial system following thermal injury Am J Physiol 179 93
42 SCHMIDT R M 1954 Über Veranderungen des retikuloendothelialen Systems bei der multiplen Sklero Arzt Wochschr 9 707
43 TEILUM G & A LINDAHL 1954 Frequency and significance of amyloid changes in rheumatoid arthritis Acta Med Scand 149 449
44 DELAUNAY A & J LEBRUN 1954 Étude du pouvoir phagocytaire des polynucléaires et des macrophages chez le rat soumis a une hypothermie expérimentale Rev Immunol 18 180
45 HOAGLAND R J 1958 Infectious mononucleosis Clin Symposia (Ciba) 10(1) 17
46 GUTMANN N & P CONSTANTINIDES 1955 Inhibition of experimental atherosclerosis by sulfated alginic acid AMA Arch Pathol 59 717
47 FEKETE G I GYERMEK & I LAZAR 1955 Studies on the storage of dextran Acta Physiol Acad Sci Hung 8 147
48 WEBURN MASON I 1958 The Reticulo Endothelial System in Growth and Tumour Formation 1/8 Kimpton London England
49 GOZSY B & L KATÓ 1956 Studies on the effects of phagocytic stimulation on microbial disease A Stimulation of phagocytic activity on monocytes against tubercle bacilli strain BCG Can J Biochem Physiol 34 571
50 BENACERRAF B C BIOZZI B N HALPERN & C STIFFEL 1955 Physiology of phagocytosis of particles by the RES RES Bull I 44
51 HELLER J H 1955 Physiologic stimulation and inhibition of the phagocytic function of the reticuloendothelial system RES Bull I 51
52 GORDON A H 1957 The use of the isolated perfused liver to detect alterations to plasma proteins Biochem J 66 255
53 TUTTLE L W & R C BAXTER 1955 The effect of polonium alpha particle irradiation upon reticuloendothelial function as measured with P[32] labelled particulate chromic phosphate Univ Rochester Atomic Energy Project UR 295
54 LAUVERT R J L BENHAMOU S NICOLLO & A LOVERDO 1958 La clearance de l'or colloïdal radio actif (Au 198) I Valeurs normales et valeurs pathologiques Rev franç etudes clin et biol III 7(?)
55 HALPERN B N G BIOZZI G PÉQUIGNOT C STIFFEL B DELALOVE & D MOLTO 1958 La cinétique de l'épuration sanguine des suspensions colloïdales comme mesure de la circulation hépatique Rev franç études clin et biol III 549
56 MABRY D S J A BASS M C DODD J H WALLACE & C S WRIGHT 1956 Differential phagocytosis of normal trypsinized and virus treated human and rabbit erythrocytes by macrophages in tissue culture J Immunol 76 54
57 SHERSTNEVA O S 1958 Dependence of the phagocytic reaction of leukocytes on their carbohydrate metabolism Bull Exptl Biol Med 45 333
58 DOBSON E L 1955 Factors controlling phagocytosis RES Bull I 47
59 TITOV K C 1956 The function of the megakaryocytes Probl Gematol Perel Krov 1 1/2 30
60 TULLIS J L & D M SURGENOR 1956 Phagocytosis promoting factor of plasma and serum Ann N Y Acad Sci 66(2) 386
61 BENACERRAF B G BIOZZI B N HALPERN C STIFFEL & D MOUTON 1957 Phagocytosis of heat denatured human serum albumin labelled with I and its use as a means of investigating liver blood flow Brit J Exptl Pathol 37 35
62 CAMPBELL R M D I CUTHBERTSON C M MATTHEWS & A S MCFARLANE 1956 Behaviour of [14]C and [131]I labelled plasma proteins in the rat Intern J Appl Radiation and Isotopes 1 66
63 TAPLIN G V C FINNEGAN P NOYES & C SIRACUSE 1954 Liver retention of intravenously injected colloidal protein in normal and Roentgen irradiated rabbits an index of phagocytic function of the reticuloendothelial system Am J Roentgenol 71 294
64 ANISIMOVA P S 1953 Proba Tamogr ... Klin Med Moskva 31
65 PEGUIRON L O JALLUT I FRECH & S NECK 1955 Method for preparation of colloidal suspensions of radioactive CIO ... Arch ... ameter RES Bull I 3) 65
66 BENACERRAF B G BIOZZI B N HALPERN & C STIFFEL 1956 A study of the phagocytic function of the reticulo endothelial system ... heat denatured human serum albumin tagged with I ... RES Bull II(2 ...)
67 WADDELL W K R P GEYER F CLARKE & F J STARE 1954 Function of the reticulo-endothelial system in removal of emulsified fat from blood Am J Physiol 177 90

68 SNELL J F & R. GARKUSCHA 1956 The effect of mucin on phagocytosis of colloidal chromic phosphate RES Bull II(2) 46
69 BIOZZI G B N HALPERN B BENACERRAF & C STIFFEL 1957 Phagocytic activity of the reticulo-endothelial system in experimental infections In Physiopathology of the Reticulo-Endothelial System 204 B N Halpern Ed Blackwell Scientific Publ Oxford England
70 REINER J M 1959 Behavior of Enzyme Systems 160 Burgess Minneapolis Minn
71 SNELL J F The Reticulo-Endothelial System II Effects of antibiotics on phagocytic functions of the system In preparation
72 HELANDER S & L E BOTTIGER 1953 On the distribution of Terramycin in different tissues Acta Med Scand 147 71
73 ANDRÉ T 1956 Studies on the distribution of tritium labelled dihydrostreptomycin and tetracycline in the body Acta Radiol. Suppl 142
74 SNELL F J R. GARKUSCHA & E L. ALLEN 1958 Radioactive oxytetracycline II Distribution in C 57 BL mice (preliminary experiments) In Antibiotic Annual 1957–1958 507 Medical Encyclopedia New York N Y
75 HOWARD J G D ROWLEY & A C WARDLAW 1958 Investigations on the mechanism of stimulation of non specific immunity by bacterial lipopolysaccharides Immunology 1(3) 181
76 ESPLIN D W & S MARCUS 1954 Relation between antibacterial action of penicillin and mouse host defenses in systemic pneumococcus infection Antibiotic & Chemotherapy 4 473
77 FEYEL CABANES T 1952 Stimulation of various elements of mesenchymal tissue by chloramphenicol Compt. rend acad sci 234 2232
78 GIUNCHI G L A SCURO & F SORICE 1953 La comparsa di anticorpi antieritrocitari in ratti trattati con vari antibiotici Poll soc ital biol sper 29 67
79 GOLDBERG L & J P SMITH 1954 The influence of aureomycin on the response of the rabbit to cholesterol feeding J Pathol Bacteriol 68 475
80 SLANETZ C A 1953 Inhibition of antibody formation by antibiotics Antibiotics & Chemotherapy 3 69
81 SKINSNES O K & R L WOOLRIDGE 1948 The relationship of biological defense mechanisms to the antibiotic activity of penicillin I The modifying influence of penicillin on the pattern of pneumococcic infection and the immune response in the protein-depleted rat J Infectious Diseases 83 78
82 SKINSNES O K 1948 The relationship of biological defense mechanisms to the antibiotic activity of penicillin II The modifying influence of penicillin on the histopathological pattern of pneumococcus infection in normal and debilitated rat and guinea pigs J Infectious Diseases 83 101
83 ZERMAN A M A PETRONELLI & P BELLINATO 1954 Il potere fagocitario antimicrobico in vitro degli istiociti e dei mielociti neutrofili ed eosinofili in alcuni casi di malattie infettive prima e dopo terapia antibiotica (penicillina streptomicina) G orn mal infettive e paras it 6 24
84 KOKHANOVSKAYA T M 1956 The influence of Biomycin and Terramycin on phagocytosis of bacteria (Breslau s and Gertner s) in vitro Antibiotiki (Russian) VI(6) 45
85 MALEK P J ROKOS M BURGER J KOLC & P PROCHAZKA 1959 The effect of antibiotics of the tetracycline groups on enzymes and the practical clinical significance thereof Antibiotics Annual 1958–1959 221 Medical Encyclopedia New York N Y
86 MALEK P J KOLC M HEROLD & J HOFFMAN 1958 Lymphotrophic antibiotics—antibilymphins Antibiotics Annual 1957–1958 546 Medical Encyclopedia New York N Y
87 HEWES C G & D F SHAY 1955 Antibody formation in the guinea pig as a result of oral administration of chlortetracycline and penicillin Antibiotics & Chemotherapy 4 101
88 PLANELES H H & N V CHUMACHENKO 1956 The influence of antibiotics on the concentration of the antibodies circulating in the blood Antibiotiki (Russian) 1 25
89 AVERIANOVA L L 1957 The influence of antibiotics on the immunological reactivity of the body J Microbiol Epidemiol Immunol 28 501
90 CONIGLIO J G & E J BELL 1957 The effect of antibiotics upon intestinal and hepatic lipases J Biol Chem 226 805
91 MARCUS S D W ESPLIN & G A HILL 1953 Effects of cortisone ascorbic acid and Pitomen on phagocytosis in mice Proc Soc Exptl Biol Med 84 565
92 DILUZIO N P 1955 Effect of X irradiation and choline on the reticulo-endothelial system of the rat Am J Physiol 181 595

93 VENKATARAMAN P R A DURIN & M T FRIEDELL 1954 Influence of cortisone and Vitamin B12 on the growth and P32 uptake of adrenal and lymphoid tissue Metabolism 3 502
94 GABRIELI E R 1951 Effect of antihistamine on the reticulo-endothelial system investigation of reticulo endothelial function with the aid of radioactive chromium phosphate Nature 168 467
95 GOTSY B & L KÁTÓ 1955 Studies on the effects of phagocytic stimulation on microbial disease III The influence of antihistamines and 1 4 dimethyl 7 isopropyl azulene on experimental tuberculosis—preliminary experiments Can J Microbiol 1 461
96 MEIER R M D A BOROFF & J H HELLER 1957 Reticulo endothelial effects of some ataractic agents Federation Proc 16 425
97 HADIDIAN Z M M MURPHY & I R MAHLER 1957 Studies of inhibitors of bacterial and other hyaluronidases II Heparin and other non specific inhibitors J Bacteriol 73 491
98 GAY K K D M DONALDSON & S MARCUS 1955 Influence of cortisone Piromen histamine and heparin on intracellular digestion by mouse peritoneal phagocytes RES Bull I(3) 75
99 CLAPPER W E & G H MEADE 1955 The effect of foreign body particles on infections in mice U S Atomic Energy Comm Health & Biol Report No AECU 3272 Technical Inform Serv Ext Oak Ridge Tenn
100 HIRSCH J G 1956 Phagocytin a bactericidal substance from polymorphonuclear leucocytes J Exptl Med 103(5) 589
101 PARFENTJEV I A 1953 A fraction of commercial yeast which protects against bacterial infection Yale J Biol Med 26 75
102 PARFENTJEV I A 1957 Prenatal protection of mice by yeast antibiotic (Malucidin) Science 125 928
103 RICCI G E CASINI L RAVAZZONI & S G CORRADINI 1958 Comportamento dei livelli serici di properdina nell uomo in seguito ad introduzione venosa di saccaromiceti Aggiorn Malattie da Infezione 4 1
104 DONETS I I 1957 Study of the effect of *Clostridium botulinum* toxin on the phagocytic properties of elements of the reticulo-endothelial system *In* Anaerobic Infections 148 Kiev U S S R
105 RAŠKOVÁ H 1958 Pharmacology of Some Toxins Czechoslovak Acad Sci Prague Czechoslovakia
106 LUDÁNY G A DOKLEN & E TOTH 1957 Die Beeinflussung der Leukozytenphagozytose mit Phlorrhizin Experientia 8 409
107 KOZLOV V A 1956 The influence of general novocaine action on staphylococcal phagocytosis in the peritoneal cavity of white mice Abstracts from Second Scientific Conf of Chita Med Inst 85 Chita U S S R
108 WEIKEL J H & L M LUSKY 1956 Pharmacology of the reticulo endothelial system I Blockade by polyvinylpyrrolidone (PVP) as measured with radiochromic phosphate in the rabbit J Pharmacol Exptl Therap 118 148
109 WESTPHAL O A NOWOTNY O LUDERITZ H HURNI E EICHENBERGER & G SCHONHOLZER 1958 Die Bedeutung der Lipoid Komponente (Lipoid A) für die biologischen Wirkungen bakterieller Endotoxine (Lipopolysaccharide) Pharm Acta Helv 33 401
110 THOMAS L 1952 Effects of cortisone and adrenocorticotropic hormones on infection Ann Rev Med 3 1
111 KASS E H & M FINLAND 1953 Adrenocortical hormones in infection and immunity Ann Rev Microbiol 7 361
112 GERMUTH F G JR 1956 Role of adrenocortical steroids in infection immunity and hypersensitivity Pharmacol Revs 8 1
113 JOHNSON J R & W N DAVEY 1954 Cortisone corticotropin and antimicrobial therapy in tuberculosis in animals and man a review Am Rev Tuberc 70 623
114 ROBINSON H J 1956 Adrenal cortical hormones and infection Pediatrics 17 770
115 THOMAS L 1955 Cortisone adrenocorticotrophic hormone and infection Bull N Y Acad Med 31 485
116 JAWETZ E 1954 Effect of cortisone on therapeutic efficacy of antibiotics in experimental infections A M A Arch Intern Med 93 850
117 AIKAWA J K & G T HARRELL 1953 Effect of cortisone acetate on experimental Rocky Mountain spotted fever in the guinea pig Proc Soc Exptl Biol Med 82 698
118 ROBINSON H J R C MASON & A I SMITH 1953 Beneficial effects of cortisone on survival of rats infected with *D pneumoniae* Proc Soc Exptl Biol Med 84 312

119 SINGER I 1954 The effect of cortisone on infections with *Plasmodium berghei* in the white mouse J Infectious Diseases 94 164
120 KASS E H & M FINLAND 1957 Adrenocortical hormones and the management of infection Ann Rev Med 8 1
121 DOUGHERTY T F & G L SCHNEEBELI 1955 The use of steroids as anti inflammatory agents Ann N Y Acad Sci 61(2) 328
122 HELLER J H 1957 Physiologic stimulation and inhibition of the phagocytic function of the reticulo-endothelial system *In* Physiopathology of the Reticulo-Endothelial System 43 B N Halpern Ed Blackwell Scientific Publ Oxford England
123 McLEOD C 1941 The mode of action of mucin in experimental meningococcus infection I The properties of mucin which influence its activity Am J Hyg 34B 41

HORMONAL INFLUENCE ON LYMPHOCYTE DIFFERENTIATION FROM RES CELLS*

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Introduction

One function of the reticuloendothelial cell is to act as the progenitor of a variety of more highly differentiated cells This function of the reticuloendothelial system (RES) has received less analytical attention than its phagocytic activities Little is known about the factors that influence differentiation and maturation of the lymphocytic elements arising from RES For some time we have been interested in the roles of hormones as they influence proliferation maturation and destruction of lymphocytes[1] Analyses of large amounts of data pointing to the ways in which certain corticosteroids exert their influence on lymphocytopoiesis have provided us with some concepts that can be subjected to test

In order that our interpretation of the experiments reported here may be understood a brief review of present knowledge of the action of cortisol on lymphatic tissue is presented The details are given elsewhere in papers and reviews[1-3] Analyses of our own data and those presented by others as well indicate that the steroid hormone cortisol inhibits mitosis[4,5] and by removing cytoplasm brings about morphologic maturation of the lymphocytes[5] If these effects progress far enough lymphocytokaryorrhexis is produced[5,6] These effects are dose-dependent[7] which indicates that there is a threshold amount of hormone in order that these lymphocytic responses can occur[7]

In turn it is also evident that lymphocytes of different degrees of maturity respond differently Thus medium sized and small lymphocytes are more susceptible to cortisol effect than immature or reticular lymphocytes[8] Therefore, the extent of hormone effect is determined by two factors the amount of hormone available and the state of maturity of individual cells acted upon For example if a constant amount of cortisol is administered the organs containing the largest population of small mature lymphocytes undergo greater involution than those containing more immature and reticuloendothelial cells[5,7,8]

Lymphocytic dissolution[9] is a direct effect of cortisol and is not dependent upon extralymphatic metabolism of the hormone Thus direct time lapse cinematographic phase microscopic studies demonstrate that when the dose of hormone and the number of cells acted upon is held constant there are graded effects on different cells of the crop studied[9] Small lymphocytes are destroyed and completely denuded of cytoplasm medium sized cells shed cytoplasm and demonstrate nuclear shrinkage but not death Reticular lymphocytes show little if any morphologic response to even large doses of hormone

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Lymphocytic Metabolism of Cortisol

It was established in our laboratory some time ago that lymphocytes are able to metabolize cortisol to cortisone[10] It also appears that cortisol is the hormone that displays the moderating influence on lymphocytopoiesis and that cortisone as such is not similarly effective It can be transformed back to cortisol which then exerts lymphocytopoietic inhibition The fact that lymphocytes have the capacity to reverse the enzymatic transformation and produce cortisol from cortisone is presented below

Thus there is a reversible enzyme system (11βOH dehydrogenase) in the same cell that can produce or inactivate the molecular structure necessary to bring about the cell s own maturation and death In a sense then the cell can control to a degree its own stepwise maturation

Data presented elsewhere[10] show that immature lymphocytes can transform cortisol (active) to cortisone (inactive) to a much greater extent than mature cells It is a reasonable assumption therefore that this is a major reason why they are able to resist the effects of cortisol The extent of this resistance probably is demonstrated best in animals given lethal doses of cortisol in which reticular lymphocytes and RES cells alone persist in the lymph nodes[6] These persistent cells then serve to repopulate the lymphatic organ by heteroplastic differentiation[6] Thus a progenitive seed bed is kept in order to maintain adequate lymphocyte population More mature lymphocytes have less capacity to transform cortisol to cortisone This may be due in part also to the fact that they are destroyed rapidly by cortisol

Cortisol then is the hormone which regulates the rate of mitosis and rate of maturation and death of lymphocytes These effects are linear and progressive in the sense that the population of cells turns over because cortisol can eliminate the more mature elements provide maturation of intermediate cells and undoubtedly bring about subtle changes in reticular lymphocytes

Methods

Preparation of lymphatic tissues As cortisol most actively destroys the most mature lymphocytes mice were treated with large amounts of this hormone for a long period of time (9 days) after which the lymphatic organs were removed and studied as described below Also it is known that thyroxin produces reticular hyperplasia of lymphatic organs[1] therefore the enzymatic ability (11βOH dehydrogenase) of thymic cells that are present after the treatment with cortisol or L triiodothyronine was studied in the following manner the thymi of mice to be studied were minced and placed in incubation flasks containing phosphate buffer *p*H 7 4 and cortisol 4C[14] or cortisone 4C[14] and incubated for a period of 3 hours at 37 C

Each tissue sample was divided in two one part was incubated with cortisol 4C[14] and the other with cortisone-4C[14] for the purpose of ascertaining the ability of the cells to interconvert these two steroids Thymi of saline treated mice of the same strain were incubated in the same manner as the controls

The extraction procedures[11] and the chemical determination and identification of the steroids isolated from the incubations were carried out as previously described[12,13]

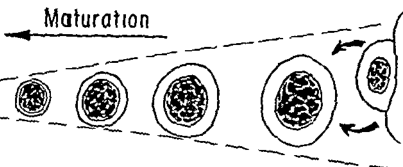
Results and Discussion

From TABLE 1 it may be seen that treatment with cortisol increased by about 246 per cent the oxidation of cortisol to cortisone, however this same tissue

TABLE 1
INTERCONVERSION OF CORTISOL AND CORTISONE BY THYMUS

Treatment	Oxidation* mμM/100 mg tissue	Reduction† mμM/100 mg tissue
Saline	0.095	0.100
Cortisol	0.329	0.128
L triiodothyronine	0.100	0.248

* Conversion of cortisol → cortisone
† Conversion of cortisone → cortisol
Incubation of 1.5 mμM/100 mg of tissue



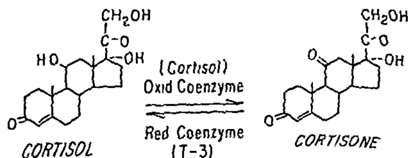


FIGURE 1 Reversible enzyme system (11βOH-dehydrogenase) in thymus

did not significantly change the conversion of cortisone to cortisol (reduction) in the same period

The opposite effect was found to occur when mice were treated with 0.25 μM triiodothyronine for four days that is a marked increase in the conversion of cortisone to cortisol was observed with no significant change in the conver-

ion of cortisol to cortisone It is well known that the reversibility of $11\beta OH$ dehydrogenase system activity is influenced by addition or depletion of reduced and oxidized coenzymes (DPN and/or TPN DPNH and/or TPNH)[11] It is suggested that thyroid hormone can make available reduced coenzyme to the enzyme system and thus favor maintenance of cortisol in the active state and in addition tend to increase conversion for cortisone (inactive) to cortisol (active) By these two mechanisms there is a greater concentration of active maturing hormone available The opposite would be the case for prolonged oversupply of cortisol (particularly) if the action of this hormone is not countered by the thyroid hormone That this is the case for growth and involution when evaluated by weight and nitrogen content of lymphatic organs has long been known[15]

It has been shown previously that L T 3 can increase the conjugation of corticosterone in the liver by increasing the reduction of ring A (Berliner and Dougherty in this monograph) it has also been demonstrated that cortisone can be reduced to dihydrocortisone faster in animals treated with thyroxin probably through an increase in the level of TPNH + H[16] Thus we can say that L T 3 can influence two completely different enzymic reactions in two different tissues by increasing the capacity of the tissue to reduce the steroid incubated that is in the thymus from cortisone to cortisol (reduction in position 11) and in the liver from cortisone to dihydrocortisone (reduction in positions 4 5)

The opposite effect occurs in thymus when the animals are treated with large amounts of cortisol[10] the cells left after the treatment can inactivate the cortisol faster probably through an increased availability in this cell of oxidized coenzyme necessary to convert cortisol to cortisone

It is therefore postulated as shown in FIGURE 1 that the lymphocyte contains a redox system ($11\beta OH$-dehydrogenase) that depending upon the concentration or availability of oxidized and/or reduced coenzymes determines the state of differentiation and rates of proliferation and maturation of lymphocytes Cortisol is the molecular form inhibiting proliferation and enhancing maturation Immaturity is maintained by cellular oxidation of cortisol The greater availability of reduced coenzyme enhances the cortisol maturing effects by inhibiting the oxidation of this compound

References

1 DOUGHERTY T F 1952 Effect of hormones on lymphatic tissue Physiol Revs 32 379-401
2 DOUGHERTY T F 1959 Adrenal cortical control of lymphatic tissue mass *In* The Study of the Kinetics of Cellular Proliferation 264-274 Grune & Stratton New York N Y
3 FRANK J A & T F DOUGHERTY 1953 Cytoplasmic budding of human lymphocytes produced by cortisone and hydrocortisone in *in vitro* preparations Proc Soc Exptl Biol Med 82 17 19
4 DOUGHERTY T F & A WHITE 1944 Influence of hormones on lymphoid tissue structure and function The role of the pituitary adrenotrophic hormone in the regulation of the lymphocytes and other cellular elements of the blood Endocrinology 35 1 15
5 DOUGHERTY T F & A WHITE 1945 Functional alterations in lymphoid tissue induced by adrenal cortical secretion Am J Anat 77 81 116
6 DOUGHERTY T F 1952 Studies of the antiphlogistic and antibody suppressing functions of the pituitary adrenocortical secretions Recent Progr in Hormone Research 7 307-330

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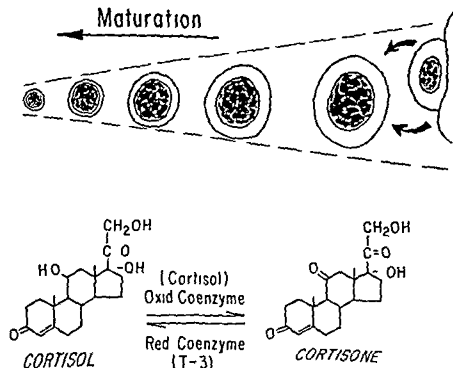


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THE RETICULOENDOTHELIAL SYSTEM, CORTISONE, AND THYROID FUNCTION: THEIR RELATION TO NATIVE RESISTANCE TO INFECTION*

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While the ultimate nature of resistance to the chronic disease tuberculosis still remains elusive, there is little doubt that one of the most decisive factors in the progress of this disease is the fate of the bacilli within the mononuclear phagocytes, the reticuloendothelial cell. In the final analysis it is the response of these cells to the ingested microorganism, with or without the synergism of body fluids and the problematical humoral or cellular antibodies, that determines whether the result is an inapparent fleeting infection, or a fulminating and fatal tuberculosis, or a long-drawn-out chronic disease with its alternating periods of remission and exacerbation. It is the purpose of this communication to integrate the host-parasite interactions of these cells and the spread of the bacilli from the portal of entry to the draining lymph nodes in untreated highly inbred natively resistant and susceptible rabbits, and the effects on these host-parasite relationships of cortisone and thyroidectomy on the one hand, and hyperthyroidism on the other, in inbred rabbits of uniform resistance.

Materials and Methods

The model of quantitative airborne inhalation infection of human-type tubercle bacilli H37Rv was used in rabbits of known different genetic resistance to tuberculosis.[1] The fate of the bacilli in the individual lesion in the lungs, in the draining lymph nodes, and in the spleen was studied, together with the correlated tissue responses at different intervals from 1 day to 4 weeks after inhalation. In addition, in untreated natively resistant and susceptible rabbits, these host-parasite relationships were investigated 2 months[2] and 1 year after infection.[3] It must be emphasized that even in the most susceptible rabbits the disease thus produced regressed and was not accompanied by systemic toxic manifestations. Similar procedures were used in the studies on the effects of cortisone administration[4, 5] and alteration of thyroid function[6, 7] on these host-parasite relationships. Needless to say, in each of these latter experiments the host-parasite relationships in untreated siblings of a given race and of a given genetic resistance were compared with those of cortisone-, triiodothyronine-, and thyroxine-treated, or thyroidectomized rabbits of the same race simultaneously exposed to the quantitative inhalation of the same aerosol of human tubercle bacilli.

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7 SANTISTEBAN G A & T F DOUGHERTY 1954 Comparison of the influences of adrenocortical hormones on the growth and involution of lymphatic organs Endocrinology 54 130–146
8 DOUGHERTY T F 1960 Lymphocytokaryorrhectic effects of adrenocortical steroids *In* The Lymphocyte and Lymphocytic Tissue Hoeber New York N Y In press
9 SCHNEEBELI G L & T F DOUGHERTY 1958 Microphotographic and microcinematographic screening method for steroid hormone effects on leukemic cells Cancer Research 2 342
10 DOUGHERTY T F M L BERLINER & D L BERLINER 1960 11β hydroxy dehydrogenase system activity in thymi of mice following prolonged cortisol treatment Endocrinology 66 550
11 BERLINER M L D L BERLINER & T F DOUGHERTY 1958 Metabolism of progesterone by adrenal tissue from patients with Cushing s syndrome and mammary carcinoma J Clin Endocrinol and Metabolism 18 109–113
12 BERLINER D L & H A SALHANICK 1956 Microchemical identification of radioactive and non radioactive steroids Anal Chem 28 1608
13 BERLINER D L O V DOMINGUEZ & G WESTENSKOW 1957 Determination of C^{14} *steroids on paper chromatograms* Anal Chem 29 1797
14 HURLOCK B & P TALALAY 1959 Microsomal 3α and 11β Hydroxy steroid Dehydrogenases Arch Biochem Biophys 80 468–470
15 WHITE A & T F DOUGHERTY 1947 Role of the adrenal cortex and the thyroid in the mobilization of nitrogen from the tissues in fasting Endocrinology 41 230–242
16 TOMKINS G M 1959 Enzymatic metabolism of corticosteroids Ann N Y Acad Sci 82(4) 836–845



FIGURE 2 (*a*) A portion of an early tuberculous focus in the lung of susceptible rabbit Cl4 5 two weeks after the quantitative inhalati n of an estimated 35 400 tubercle bacilli Numerous tubercle bacilli many of them in the form of very dense skeins swarm in the intra alveolar ph gocytes Ì envascular accumulation f m nonuclears with some l acilli is seen in the upj e ight corner ×465 (*b*) An early tuberculous focus in the lung of resistant rabbit IIII 4-7o two weeks after the quantitative inhalation of 35 700 microorganisms Mod erate numbe s of tubercle bacilli are seen in the intra alveolar phagocytes Only one small skei indicat d by the arrow can be seen in the upper alveolar pace which is infiltrated by umerous polymorphonuclear leukocytes ×465 (*c*) P imitive les on in untreated rabbit CaS 10 t o weeks after quantitati e inhalation of tubercle bacilli The a rows indicate the me ger numbers of mic oorganisms ithin the phagocytes ×360 (*d*) Primiti e lesion in the lung of C 5 9 a cort sone treated littermate of CaS 10 shown in *c* two weeks after inha lation Numerous intracellular bacilli are found within the phagocytes ×360

Results

Host Parasite Relationships in Untreated Natively Resistant and Susceptible Rabbits

Figure 1 illustrates the fate of human type tubercle bacilli in the lungs of the natively susceptible race C and of the natively resistant race III at different intervals after quantitative inhalation of human type tubercle bacilli. On the ordinate are plotted logarithmically the ratio between the number of bacilli recovered from and the number seeded in the lungs at different intervals following infection. It is evident that early in the course of the infection and

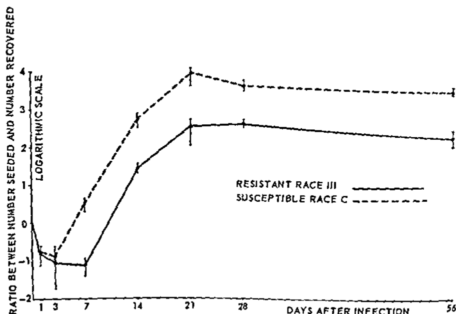


Figure 1. Population changes of human type tubercle bacilli (H37Rv) in the lungs of natively resistant and susceptible rabbits at different intervals after quantitative airborne infection (treated culture).

long before specific acquired resistance becomes manifest inbred natively resistant rabbits inhibit the accumulation of inhaled human type tubercle bacilli in the lungs 20 to 30 times more effectively than susceptible rabbits. Figure 2a and b demonstrate that at the height of bacillary multiplication in the lungs of both races 2 weeks after infection the reticuloendothelial cells (mononuclear phagocytes) of the resistant animals contain very many fewer organisms in their cytoplasm than the cells in the susceptible animals. Specific acquired immunity develops at the same time in both races but its level is superimposed on and quantitatively determined by the initial native resistance. Thus even 1 year after infection when the disease had regressed in both races the residual viable bacilli are still twentyfold more numerous in the susceptible than in the resistant animal. The degree of healing in the

to a variety of infections there is no agreement on the mode of action of this hormone in this relation The most widely held hypothesis claims that the anti inflammatory effect of the steroid enhances the disease and its spread[3] In the infection of rabbits by the quantitative inhalation of human type bacilli however it was demonstrated that far from enhancing the spread of the bacilli from the portal of entry to the draining lymph nodes cortisone markedly retard their dissemination[4] This is illustrated in FIGURE 4 The lowered

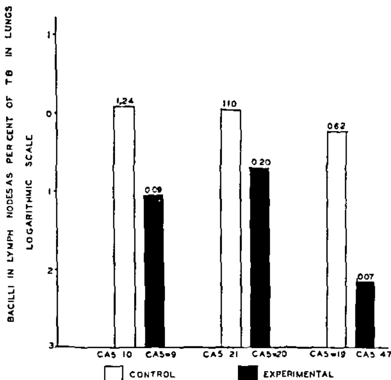


FIGURE 4 The percentage of human type bacilli from the lungs that were recovered from the draining lymph nodes of cortisone treated and control susceptible race CA rabbits ? weeks after infection by inhalation

resistance of the steroid treated animals is apparently associated with a deprivation of the reticuloendothelial cells (mononuclear phagocytes) of much of their innate capacity to inhibit the growth of tubercle bacilli within their cytoplasm This is illustrated in FIGURE 2c and d Furthermore cortisone retards the maturation of tubercle bacilli infested mononuclears into epithelioid cells[6] which again testifies to the reduced capacity of these reticuloendothelial cells to inhibit the growth of the bacilli in their cytoplasm Similar observations by Thomas[9] Kass *et al*[10] and Cremer and Watson[11] suggest that steroids impair the function of the reticuloendothelial system (RES) in eliminating or detoxifying bacteria certain of their products and other phagocytized material That

2 races at this time is of a corresponding order[2] It is noteworthy that the maturation of mononuclear phagocytes into epithelioid cells, a transformation that is uniformly associated with the diminution of the ingested bacilli, is much more rapid in the natively resistant rabbit[2] indicating that the acquired immunity is conditioned by the native resistance and is more effective in genetically resistant animals

Significantly the spread of the bacilli from the portal of entry (the lung) to the draining lymph nodes is contrary to expectation, enhanced in the resistant rabbit FIGURE 3 illustrates this difference Thus while 20 per cent of the bacilli present in the lung were recovered from the lymph nodes of the resistant

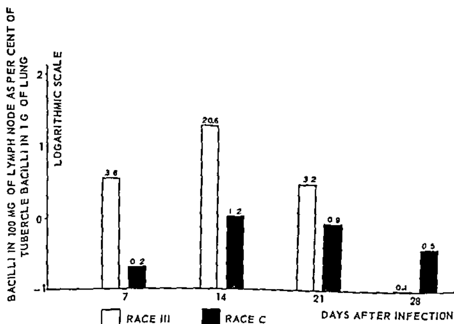


FIGURE 3 The percentage of human type bacilli from the lungs that were recovered from the draining lymph nodes in resistant race III and susceptible race C rabbits

animal 2 weeks after infection only 1 per cent of the bacilli present in the lungs of the susceptible animal were recovered from its draining nodes Four weeks after infection however the bacilli in the nodes of resistant rabbits had been reduced to one twenty sixth of the number present in these nodes on the third week after infection whereas the bacilli in these nodes in the susceptible animal were reduced by only one half in the same interval It is evident that while the bacilli in the resistant animal are disseminated from the portal of entry to a greater degree than in the susceptible their growth is much more effectively inhibited in the metastatic foci of the former

Host Parasite Relationships in Cortisone Treated Rabbits

While it is generally accepted that the administration of certain amounts of cortisone and its variants markedly reduces the resistance of many species

slight FIGURE 5 demonstrates that the increased resistance afforded by triiodothyronine is due to the marked suppression of the accumulation of tubercle bacilli in the lung of the hormone treated animals FIGURE 6 hows that the tran port of bacilli from the portal of entry to the draining lymph nodes is enhanced in the hyperthyroid rabbits

Similar observations were made on some of these races treated with L thyroxine The reduction of bacillary accumulation in the lung produced by

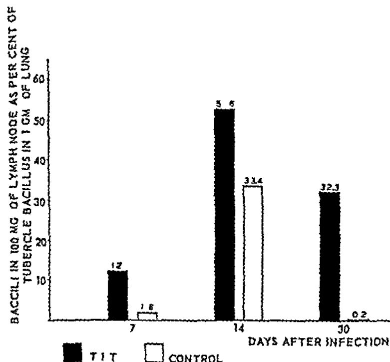


FIGURE 6 The percentage of human type tubercle bacilli from the lungs that were recovered from the draining lymph nodes of TIT treated and control race AD rabbits of intermediate resistance

thyroxine is illustrated in FIGURE 7 and the inhibition of bacillary growth within the reticuloendothelial cells of the lung is depicted in FIGURE 8a and b

Thus hyperthyroidism increases the resistance of a given race in the same manner as natively resistant animals restrict the disease more than the natively susceptible animals In both instances the increased resistance is brought about by an augmentation of the inhibitory capacity of reticuloendothelial cells against the accumulation of the bacilli within their cytoplasm and consequently a more rapid maturation of epithelioid cell Associated with this increment in resistance in the hyperthyroid rabbit is an enhancement of the spread of the bacilli from the site of invasion a relationship similar to that in natively resistant rabbits

a correspondence exists between the direct action of adrenocortical hormones on cultured cells and their action in the organism has been demonstrated by Grossfeld[1]

The difference in host parasite relationships between a cortisone treated rabbit and an untreated sibling of the same genetic resistance is of a imilar general nature as that between an untreated natively susceptible and untreated natively resistant animal In both instances reduced resistance is associated with a reduced ability of RES to inhibit the intracellular growth of the bacilli

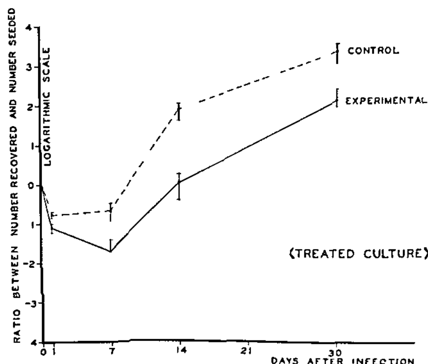


FIGURE 5 The effect of TIT on population changes of human type bacilli (H37Rv) in the lungs of AD rabbits at different intervals after quantitative airborne infection

a retardation in the maturation of epithelioid cells and a diminution in the spread of the bacilli from the site of invasion

Host Parasite Relationships in Triiodothyronine and Thyroxine Treated and in Thyroidectomi ed Rabbits

Since hyperthyroidism increases the metabolic cellular activity an investigation into the effect of thyroid function on native resi tance was undertaken It was found that hyperthyroidism induced by I triiodothyronine (TIT) or L thyroxine markedly increased the native resistance to the inception and progress of the disease in 4 different inbred races of rabbits chiefly of intermediate genetic resistance to the disea e[6] The increment in resistance afforded by TIT to the most susceptible race in our colony (C) was discernible although

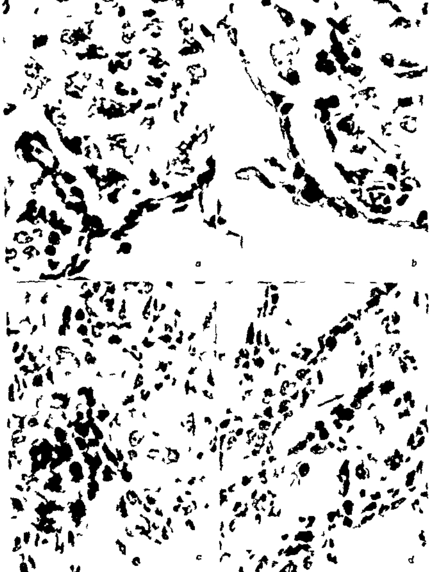


FIGURE 8 (*a*) Primitive tuberculous focus in control rabbit III IIIC4-11 two weeks after the inhalation of 9 000 bacilli Note the numerous tubercle bacilli within alveolar phagocytes and the single skein of microorganisms near the lower edge of the alveolar space The bacilli in this lung had multiplied two thousandfold over those seeded ×747 (*b*) Primitive tuberculous focus in the thyroxine treated rabbit III IIIC4 10 a sibling of III IIIC4 11 shown in *a* 2 weeks after the inhalation of 18 000 bacilli A rare bacillus within an alveolar phagocyte is indicated by the arrow The bacilli in this lung had multiplied 61 times over those seeded × [illegible] (*c*) Primitive tuberculous focus in the lung of intact rabbit III A5-4 two weeks after inhalation of 8 800 bacilli An occasional alveolar phagocyte contains some bacilli The bacilli in this lung had multiplied ninety sixfold over the number seeded There is extensive interstitial mononuclear infiltration to the left of the alveolar space ×747 (*d*) Primitive tuberculous focus in the lung of thyroidectomized rabbit III A5-3 a sibling of III A5-4 shown in *c* two weeks after the inhalation of 7 900 bacilli There are numerous bacilli within the alveolar phagocytes A very dense skein of tubercle bacilli within one such phagocyte is indicated by the arrow The bacilli in this lung had multiplied five hundred and sixty-eight fold over the number seeded There is very slight thickening of the septum surrounding this alveolar space ×747

The converse of the above is produced by suppressing thyroid function with propylthiouracil or thyroidectomy.[7] FIGURE 9 shows that the accumulation of tubercle bacilli in the lungs of the thyroidectomized IIIA rabbits is 10 times greater than in their intact siblings exposed simultaneously to the quantitative inhalation of the same aerosol of human bacilli. FIGURE 8c and d shows that thyroidectomy deprives the reticuloendothelial cells of much of their innate capacity to inhibit the growth of the bacilli within their cytoplasm. Associated with this reduction of inhibition of intracellular multiplication is a retardation

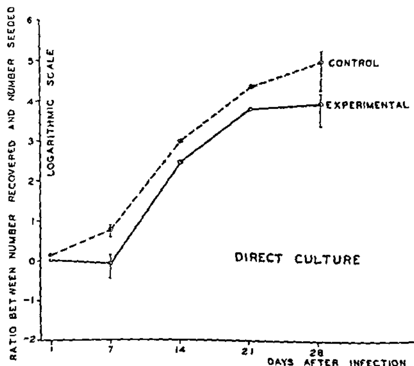


FIGURE 7. The effect of L-thyroxin on population changes of human type bacilli (H37Rv) in the lungs of III IIIC rabbits at different intervals after quantitative airborne infection.

in the maturation of epithelioid cells and a marked diminution of the transport of the bacilli from the portal of entry to the draining lymph node, as contrasted with their behavior in intact siblings of the same genetic resistance, as illustrated in FIGURE 10.

Thus, thyroidectomy induces host parasite relationships similar to those obtaining in untreated natively susceptible rabbits and in cortisone treated animals. In all these instances lowered resistance is associated with a reduced capacity of the reticuloendothelial cells to suppress the growth of the bacilli in their cytoplasm, a slower maturation of epithelioid cells, and a retardation in the spread of the bacilli from the portal of entry.

While there is no strict parallel between the degree of native resistance and the capacity for antibody formation there is a tendency in this direction Thus the very susceptible race FC rabbit produces very low antibody titers as compared with the more resistant races AD or III[7] Thus the increased inhibition of bacillary accumulation within the reticuloendothelial cells is

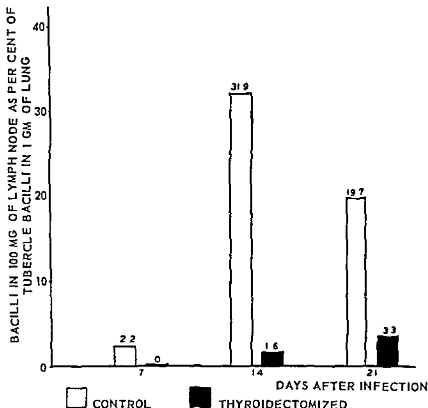


FIGURE 10 The percentage of human type bacilli from the lu gs that were recovered from the dra ning lymph nodes of thyroidectomized and control race IIIA rabbits of intermediate res stan e

generally associated with an increased capacity for antibody formation against antigens in general If antibody formation is considered a function of reticuloendothelial cells then these data would lend additional support to the concept that increased resistance whether afforded by the native constitution or induced by hyperthyroidism is an expression of the increased activity of these cells and conversely reduced resistance whether determined by genetic factors or following the administration of cortisone or the induction of hypothyroidi m is a consequence of the reduced reticuloendothelial cell activity

Phagocytic Activity and Antibody Production of Reticuloendothelial Cells in Rabbits of Different Degrees of Native Resistance, in Cortisone Treated and in Hyperthyroid and Hypothyroid Rabbits

Contrary to expectation, natively highly resistant race III rabbits cleared their blood stream of colloidal carbon much more slowly than did susceptible rabbits of the C or FC strains This clearance was measured by the method of Benacerraf[13] with uniform carbon particles kindly furnished us by J H Heller of the New England Institute for Medical Research, Ridgefield Conn and B Benacerraf of New York University New York, N Y, and adminis-

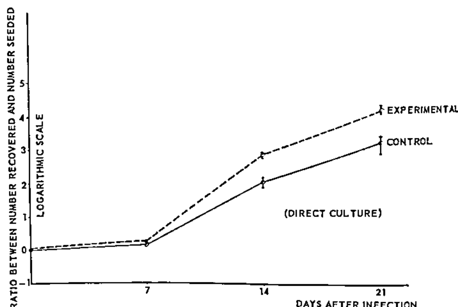


FIGURE 9 The effect of thyroidectomy on population changes of human type bacilli (H37Rv) in the lungs of race III A rabbits at different intervals after quantitative airborne infection

tered intravenously in doses of 5 mg /100 gm body weight Cortisone uniformly retarded the blood clearance while hyperthyroidism induced by TIT and hypothyroidism induced by thyroidectomy appeared to have no effect Thus there was no constant relationship between the phagocytic avidity of the RES for these particles and the fate of tubercle bacilli ingested by reticuloendothelial cells

However there seems to be a fairly constant correlation between resistance to infection and the capacity to form antibodies Hyperthyroidism induced by TIT tends to increase antibody formation as indicated by the amount of antibody nitrogen formed early after primary or secondary immunization with bovine serum albumin On the other hand thyroidectomy definitely and significantly retarded and depressed antibody formation to this antigen It is well known that cortisone suppresses antibody production by the RES

et al[25] first observed that thyroxine reduces the half life of γ globulin in rabbits from 4 6 to 3 2 days It is understandable therefore that the rate of decline of antibody nitrogen in the serum after it reaches its peak is more rapid in the hyperthyroid than in the euthyroid animal Conversely, our observations on the reduction in antibody formation by thyroidectomy have also been confirmed by Tripani and his co workers who also found that the half life of passively administered antibody is twice as long in thyroidectomized as in euthyroid rabbits[26] Correspondingly the decay of antibody in the serum after reaching its height is slower in the thyroidectomized than in the intact rabbit[7] That cortisone diminishes antibody formation is generally accepted It is thus seen that there is a close correlation between resistance and the antibody forming capacity of reticuloendothelial cells

Contrary to expectation the spread of the bacilli from the pulmonary portal of entry to the draining nodes is greater in the natively resistant than in the natively susceptible rabbit Correspondingly the spread of the bacilli from the site of invasion to these nodes is enhanced by hyperthyroidism and reduced by cortisone treatment or thyroidectomy These observations are in line with the concept of Miles and Miles[26] that one mechanism by which resistance is increased is the diminution of the local effects of noxious agents by dispersing them among humoral or cellular protective entities beyond the site of entry I pointed out many years ago[7] that the allergic inflammation in immunized rabbits accelerates the spread of the bacilli from the site of reinfection

Obviously the spread of bacilli from the portal of entry to the draining lymph nodes whether free or within phagocytes is controlled by the lymph flow which in turn is influenced by the degree of inflammation The more intense the nonnecrotizing inflammation the greater the lymph flow and consequently the greater the bacillary transport While there is some relation between the non specific inflammatory irritability and resistance it is not constant Thus cortisone and thyroidectomy both reduce inflammation The inflammatory response to turpentine is greater in the resistant rabbit race III than in the susceptible race C However no evidence is at hand to indicate that the inflammatory irritability in hyperthyroid rabbits is greater than in euthyroid animals but the spread of the bacilli from the portal of entry to the draining nodes is greater in the former It is postulated that the increased inhibition of bacillary accumulation within the reticuloendothelial cells characteristic of increased resistance may be associated with the release of inflammatory irritants possibly derived from the surface of the bacteria[28] which increase lymph flow and thus enhance the dissemination of the infectious agent and its dilution in the sense of Miles and Miles[6]

*At first glance the increased resistance afforded rabbits quantitatively in*fected by inhalation of human tubercle bacilli appears to be at variance with a number of observations on hyperthyroid mice in which the mortality from a variety of acute infections including tuberculosis was increased by TIT or *thyroxine*[29-31] *However as pointed out by Smith and Dubos*[3] *this increased* mortality was not due to an enhancement of bacterial proliferation but rather to a possible increased susceptibility of the hyperthyroid mice to toxins and other bacterial products In fact it has since been demonstrated by Melby

Discussion

It seems clear from the foregoing that there are certain host parasite relationships that characterize resistance and certain others that characterize susceptibility. Increased resistance, whether due to constitutional factors or to added thyroid treatment, is associated with an enhanced capacity of the reticuloendothelial cells to inhibit the multiplication of the bacilli in their cytoplasm *in vivo*, a more rapid maturation of reticuloendothelial cells into epithelioid cells, a generally increased capacity for antibody formation, and a greater dissemination of the bacilli from the portal of entry in the lung to the draining lymph nodes. On the other hand susceptibility, whether due to inherent genetic factors or induced by thyroidectomy or the administration of cortisone, is associated with a reduced capacity of the reticuloendothelial cells to inhibit the growth of the bacilli in their cytoplasm, a retardation in the maturation of epithelioid cells, a reduced capacity for antibody formation, and a diminished spread of the bacilli from the site of invasion to the draining nodes.

Most of the differences in the host parasite relationships between resistant and susceptible states appear to stem from differences in the degree of activity of the reticuloendothelial cells, the greater the resistance, the greater the physiological activity. It is noteworthy therefore that the increased resistance to reinfection was associated as early as 1939[14] with a nonspecific increment of the physiological activity of the reticuloendothelial cells as evidenced by their increased *in vitro* phagocytic activity for nonspecific particles and their increased propensity for division in response to stimuli applied *in vivo*. That phagocytes derived from immunized animals without the aid of humoral antibodies have a greater capacity to inhibit intracellular growth of tubercle bacilli than those derived from normal rabbits has been demonstrated by us with the *in vivo* eye chamber technique and confirmed in tissue culture studies with isolated cells by a number of observers for tubercle bacilli and *Brucella* infection[15-18] although not by all. No studies have yet been reported on whether reticuloendothelial cells derived from natively resistant animals inhibit the growth of tubercle bacilli *in vitro* to a greater degree than such cells derived from susceptible animals, nor is there authentic evidence that macrophages derived from cortisone treated animals suffer from a reduced capacity to inhibit the growth of bacteria in their cytoplasm *in vitro*. However Hsu and Kapral[19] seem to have found that mononuclear phagocytes derived from guinea pigs treated with TIT suppress the growth of virulent human type tubercle bacilli *in vitro* to a much greater degree than those derived from untreated animals.

That the maturation of mononuclear phagocytes into epithelioid cells is associated with the diminution of the ingested bacteria has been long established by us, and no evidence against this view has been presented.

That genetic resistance is associated with an increased capacity for antibody formation has been reported by numerous investigators as well as by ourselves.[20-23] Long and Shewell[3] have reported that thyroxine markedly increased the production of diphtheria antitoxin in guinea pigs. Hyperthyroidism induced by TIT increases antibody formation against bovine serum albumin.[7] This observation has been confirmed by Trapani *et al.*[24] Dixon

hoid cells of mononuclear phagocytes that had ingested tubercle bacilli Reduced resistance whether native or induced by cortisone or thyroidectomy is associated with a retardation in the maturation of mononuclear phagocytes into epithelioid cells Antibody production tends to be increased in natively resistant as compared with natively susceptible animals Hyperthyroidism has a similar effect while corti one and thyroidectomy definitely retard and depres antibody form ition

In contrast to similar experiments done in several other laboratories the infection u ed in the e studies in the rabbit leads to regressive lesions without generalized lethal toxic phenomena Under these conditions hyperthyroidi m increases re i tance The reduction in survival of hyperthyroid mice with acute infections is discu ed and i interpreted as the expression of the increased lethal effects of endotoxins in thi state

It is concluded that resistance to tuberculosis is to a great extent a function of the physiological activity of the reticuloendothelial cells that the degrees of native resistance are determined by the level of activity of these cells that hyperthyroidism increases resistance by increasing the activity of these cells and that thyroidectomy or the administration of corti one lowers resistance by depressing the function of these cells

References

1 Lurie M B S Abramson & A G Heppleston 1952 On the re ponse of g netically resistant and suscep til le rabl its to the quant tative inhalati n of human type tubercle bacilli and the nature of res stance to tuberculos s J Exp tl Me l 95 119

2 Lurie M B I Zappa odi & C Tickner 1955 On the nature of genetic resistance to tuberculosis in the light of tl e host parasite relationships in natively resistant an l susceptible rabbits Am Rev Tuberc 72 297

3 Lurie M B & P Zappasodi The ultimate fate of natively resistant and susceptil le ral l its infecte l with human type tubercle bacilli and the natu e of acqui ed resistance To be pul lished

4 Lurie M B P Zappasodi A M Dannenberg Jr & F Cardona Lynch 1953 The effect of c rtisone and ACTH on the pathogenesis of tubercul sis Ann N Y Acad Sci 56(4) 7/9

5 Lurie M B & P Zappasodi 1955 On the mod of action of cortisone on the path ogenesis of tul e culosis and its impl cations for the nature of genetic res stance to the disease Symposium on Experimental Tubercul sis 246–260 Ciba Fou dation Churchill London England

6 Lurie M B I Zappasodi I S Levy & P G Blaker 1959 On the rol of the thyroid in native re i tance to tube culos s I The effect of hyperthyroidism Am Rev Tuberc Pulm nary D seases 79 152

7 Lurie M B I Zappa odi R C Blaker & R S Levy 1959 On the role of the thyr id in native esistance to tube culos s II The effect of hyp othyroi lism The mode of action f thyroid horm nes *Ibid* 79 180

8 Germuth F K Jr 1956 The ole f adrenoco tical stero ds in infection immunity and hypersensit ty Pharmacol R v 8 1 24

9 Thomas L 1953 Cortisone an l nfection Ann N Y Acad Sci 56(4) 799

10 Kass F H Q M Geiman & M Finland 1953 Ol se vati ns on adrenal cortical horm nes in p neumococcal and nfluenza vi al infect ons and in malaria The effect of ACTH n l o ti upon infection and esistance 166–167 C lumbia Univ Press N v Y k N Y

11 Cremer N & D W Watson 1957 Influence of stress on the distribution of endo toxi i RES determined by fl o scein ant body technic Proc Soc E ptl Biol M d 95 510

12 Cre feld H 1959 Act f adrenoco tical ste o ds on cultu e l cells Endocrin ol gy 65 777

13 Benacerraf B & M M Sebestyen 1957 Effect of bacterial e dotoxins on the retic loend th li l system Federation P oc 16 860

14 Lurie M B 1939 Studies on the mechan sm of immunity in tuberculosis The mo

and Spink[33] and Bradley and Spink[34] that hyperthyroidism enhances the lethal action of endotoxins in mice, hence the greater mortality of hyperthyroid mice from acute infection The model infection under scrutiny in the present study does not involve the death of the animal but the intimate host parasite relations of the individual lesions that tend to regress and are not associated with lethal toxic phenomena

Of great interest in this relation are the observations of Halpern and his co-workers[35] on mice Suter s studies in guinea pigs[36] and particularly tho e of Howard *et al* in mice[37] All of the e investigators have shown that in animals vaccinated with BCG or treated with zymosan[38] the lethal toxicity of endotoxin is increased one hundredfold At the same time however there is evidence of activation of the RES, not only from the standpoint of its capacity to clear the circulation of carbon particles but even more significantly, from the standpoint of its affording increased resistance against the multiplication of bacteria within its reticuloendothelial cells Thus a parallel is established between hyperthyroidism and BCG vaccination, both increase the lethal effects of endotoxins yet both increase the bacteriostatic activity of the RES cells On the other hand corti one, which suppresses the bacteriostatic effects of the reticuloendothelial cells can protect mice from the lethal effects of endotoxin[39-41] It is well known that hyperthyroidism potentiates the physiological effects of epinephrine[42] It is also generally held that the lethal effects of endotoxin are partly due to its profound effects on the vascular system presumably by changing the reaction of smooth muscle to epinephrine and norepinephrine[43] Whether the action of these or other vasoactive drugs can explain how BCG vaccination or the induction of granuloma by zymosan increases toxicity to endotoxin or conversely why cortisone is protective against endotoxin and how the function of the RES mediates these effects are problems that remain to be determined

Summary

Early in the course of infection and long before specific acquired resistance develops inbred natively resistant rabbits inhibit the accumulation of inhaled human type tubercle bacilli within the pulmonary reticuloendothelial cells (mononuclear phagocytes) 20 to 30 times more effectively than susceptible animals Significantly the pread of the bacilli from this portal of entry to the draining lymph nodes is enhanced contrary to expectation in the resistant rabbit Cortisone deprives these phagocytes of much of their innate capacity to inhibit the multiplication of the bacilli in their cytoplasm and thus markedly lowers resistance Cortisone treatment also is associated with a retardation in the transport of the bacilli from the site of invasion The e host parasite relationships are characteristic of untreated susceptible rabbits Hyperthyroidism induced by thyroxine or TIT markedly suppresses the accumulation of the bacilli within the alveolar phagocytes increases resistance and enhances the lymphatic spread of the bacilli as in untreated natively resistant rabbits Thyroidectomy like cortisone enhances the accumulation of the bacilli within the mononuclear phagocytes lowers resistance and retards the dissemination of bacilli from the portal of entry Increased resistance whether native or induced by hyperthyroidism is associated with a rapid maturation into epithe

RETICULOENDOTHELIAL SYSTEM AND PASSIVE TRANSFER OF ENDOTOXIN TOLERANCE

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The peculiar state of heightened non specific resistance that characterizes the endotoxin tolerant animal has been investigated intensively in recent years The effects of bacterial endotoxins and the development of tolerance upon continued daily administration have been admirably reviewed recently by Thomas[1] and by Bennett and Cluff[2] More recently the induction by endotoxin of resistance to hemorrhagic[3,5] and traumatic[4] shock and to experimental infection[6-8] has been described

The evidence to date strongly supports the hypothesis that enhanced activity of the reticuloendothelial system (RES) is the basis of endotoxin tolerance Beeson[9] demonstrated by determining residual circulating pyrogen that the tolerant rabbit clears intravenously administered endotoxin faster than does the normal rabbit Using Cr^{51} labeled endotoxin Braude *et al*[10] also showed this enhanced clearance of toxin from the circulation in tolerant animals Intravenous injection of one of a variety of colloids known to block the RES abolishes a previously induced tolerance to endotoxin or to shock[9,11,14] Furthermore pretreatment with colloidal suspensions renders normal animals exquisitely sensitive to a first dose of endotoxin[11,15,16] or to shock[14] blocking RES uptake of the toxin[15]

Biozzi *et al*[19] and Benacerraf and Sebestyen[0] have traced the time course of change in RES activity subsequent to a single intravenous injection of endotoxin showing that during the first few hours there is a depression of carbon clearance followed later by an exalted RES activity These changes roughly parallel the time course of increased susceptibility and the then heightened resistance that occurs after such treatment with endotoxin[6-8,1,22]

Some of the effects of bacterial endotoxin can be obtained with indifferent colloids[13,14] Benacerraf and Sebestyen[0] have noted the possible relationship between the enhanced RES activity they found after zymosan treatment and the increased resistance to infection[23] after such treatment Work from the laboratories of Zweifach *et al*[13] and of Fine *et al*[14] has demonstrated that although susceptibility to shock or to endotoxin is greatly increased for a few hours after administration of an indifferent colloid (in the same way that susceptibility to a second dose of endotoxin is increased shortly after the first) suitable pretreatment with a colloid can enhance resistance to shock or to endotoxin (just as an endotoxin tolerant animal is resistant) The important difference between the bacterial endotoxins and the other colloidal substances is the specificity of the former what is done by endotoxin in a dose of a few micrograms requires many milligrams of the other colloids This is not attributable to particle size for the carbon suspension for example has a mean particle size[4,26] of 19 to 25 mμ and Westphal s[6] very highly purified lipopolysaccharide endotoxin a particle size of 10 mμ This small difference in size would result in only about a twentyfold difference in number of particles per

bilization of mononuclear phagocytes in normal and immunized animals and their relative capacities for division and phagocytosis J Exptl Med 69 579
15 LURIE M B 1942 Fate of tubercle bacilli ingested by mononuclear phagocytes derived from normal and immunized animals J Exptl Med 75 247
16 SUTER E 1953 J Exptl Med 97 235
16a BERTHRONG M & M A HAMILTON 1959 Am Rev Tuberc 79 221
17 POMALES LEBRON A & W R STINEBRING 1957 Proc Soc Exptl Biol Med 94 476
18 HOLLAND J J & M J PICKETT 1958 A cellular basis of immunity in experimental *Brucella* infection J Exptl Med 108 343
19 HSU H S & F A KAPRAL The suppressed multiplication of tubercle bacilli within macrophages derived from triiodothyronine treated guinea pigs Am Rev Respir Diseases In press
20 LURIE M B I ZAPPASODI E CARDONA LYNCH & A M DANNENBERG JR 1952 The response to the intracutaneous inoculation of BCG as an index of native resistance to tuberculosis J Immunol 68 369
21 LEWIS P A & D LOOMIS 1928 J Exptl Med 47 437
22 IPSEN J 1959 Difference in primary and secondary immunizability in inbred mice strains J Immunol 83 448
23 LONG D A & J SHEWELL 1955 Brit J Exptl Pathol 36 351
24 TRAPANI I L A LEIN & D H CAMPBELL 1959 The effect of thyroidectomy and thyroxine treatment on the immune response in rabbits Federation Proc 18 161
25 DIXON F J D W TALMAGE P H MAURER & M DEICHMILLER 1952 The half life of homologous gamma globulin (antibody) in several species J Exptl Med 96 313
26 MILES A A & E M MILES 1958 J Pathol Bacteriol 76 21
27 LURIE M B 1936 On the mechanism of immunity in tuberculosis the host parasite relationships under the conditions of a localized agar focus of infection and the generalization of the disease in normal and immunized rabbits J Exptl Med 63 923
28 RIBI E & G L LARSON 19 8 Proc Soc Exptl Biol Med 98 263
29 DUBOS R J 1955 Effect of metabolic factors on susceptibility of albino mice to experimental tuberculosis J Exptl Med 101 59
30 NUTTER J E C L GEMMIL & Q N MYRVIK 1958 Influence of triiodothyronine on the course of pneumococcosis and tuberculosis in mice Federation Proc 17 525
31 MURPHY W H JR A L WIEMS & D W WATSON 1958 Impairment of innate resistance by triiodothyronine Proc Soc Exptl Biol Med 99 213
32 SMITH J M & R J DUBOS 1956 The effect of dinitrophenol and thyroxine on the susceptibility of mice J Exptl Med 103 119
33 MELBY J C & W W SPINK 1959 Enhancement of lethal action of endotoxin in mice by triiodothyronine Proc Soc Exptl Biol Med 101 546
34 BRADLEY M G & W W SPINK 19 9 Acute hepatic necrosis induced by *Brucella* infection in hyperthyroid mice J Exptl Med 110 [illegible]
35 HALPERN B N G BIOZZI J HOWARD C STIFFEL & D MOUTON 1958 Exaltation du pouvoir toxique d *Eberthella typhosa* tuée chez la souris inoculée avec le BCG vivant Relation entre cette augmentation de la susceptibilité et l état fonctionel du système reticulo endothélial Compt rend soc biol 152 [illegible]
36 SUTER E G E ULLMAN & R G HOFFMAN [illegible] Sensitivity of mice to endotoxin after vaccination with BCG Proc Soc Exptl Biol Med 99 16
37 HOWARD J G G BIOZZI B N HALPERN C STIFFEL & D MOUTON 1959 The effect of *Mycobacterium tuberculosis* (BCG) infection [illegible] of mice to bacterial endotoxin and *Salmonella enteritidis* infection Brit J Exptl Path 40 281
38 BENACERRAF B C J THORBECKE & D JACOBY [illegible] Effect of Zymosan on endotoxin toxicity in mice Proc Soc Exptl Biol Med 100 [illegible]
39 DUFFY B J JR & H R MORGAN 1951 ACTH [illegible] aggravation or suppression of the febrile response of rabbits [illegible] Soc Exptl Biol Med 78 687
40 BENNETT I L JR & P B BEESON 19 3 The [illegible] reactions of rabbits to bacterial endotoxins with particular [illegible] Bull Johns Hopkins Hosp 93 290
41 PERRY L J D S SMYTHE & I G YOUNG 19 [illegible] on metabolism I Carbohydrate depletion and the [illegible] J Exptl Med 110 38[illegible]
42 BREWSTER W R J P ISAACS P F OSGOOD & T L KING [illegible] 13 1
43 ZWEIFACH B W B BENACERRAF & L THOMAS 19 [illegible] the vascular manifestations of shock produced by endotoxin [illegible] J Exptl Med 106 385

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The peculiar state of heightened nonspecific resistance that characterizes the endotoxin tolerant animal has been investigated intensively in recent years. The effects of bacterial endotoxins and the development of tolerance upon continued daily administration have been admirably reviewed recently by Thomas[1] and by Bennett and Cluff.[2] More recently, the induction by endotoxin of resistance to hemorrhagic[4,5] and traumatic[3] shock and to experimental infection[6-8] has been described.

The evidence to date strongly supports the hypothesis that enhanced activity of the reticuloendothelial system (RES) is the basis of endotoxin tolerance. Beeson[9] demonstrated, by determining residual circulating pyrogen, that the tolerant rabbit clears intravenously administered endotoxin faster than does the normal rabbit. Using Cr^{51} labeled endotoxin, Braude *et al.*[10] also showed this enhanced clearance of toxin from the circulation in tolerant animals. Intravenous injection of one of a variety of colloids known to block the RES abolishes a previously induced tolerance to endotoxin or to shock.[9,11-14] Furthermore, pretreatment with colloidal suspensions renders normal animals exquisitely sensitive to a first dose of endotoxin[11,15-17] or to shock,[14] blocking RES uptake of the toxin.[18]

Biozzi *et al.*[19] and Benacerraf and Sebestyen[20] have traced the time course of change in RES activity subsequent to a single intravenous injection of endotoxin, showing that during the first few hours there is a depression of carbon clearance followed later by an exalted RES activity. These changes roughly parallel the time course of increased susceptibility and the then heightened resistance that occurs after such treatment with endotoxin.[6-8,19,22]

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unit weight (on the reasonable assumption of a twofold difference in density) which hardly compares with the many thousandfold difference in effectiveness as regards the RES. Most endotoxin preparations, less highly purified than Westphal's, almost assuredly have a larger particle size.

One particularly discordant fact remains: the increased susceptibility to a second dose of endotoxin, shown originally in Fine's laboratory[1] and confirmed by others including my associates and myself, lasts only about four hours after the first dose. With similar doses of endotoxin the RES depression following a first dose,[19,20] as measured by carbon clearance, lasts much longer. It appears that resistance to endotoxin is not depressed at the time of maximal RES depression and that heightened resistance to the second dose of endotoxin occurs before demonstrable enhancement of RES function. The matter is under investigation in our laboratory.

Our recent findings that tolerance to the lethality of homologous and heterologous endotoxin in the mouse[77] and to the pyrogenic effect of endotoxin in the rabbit[78] can be achieved by passive transfer of plasma or serum of endotoxin tolerant donors, suggested studies on RES function in recipient animals made tolerant by passive transfer. If indeed there is a causal relationship between enhanced RES function and endotoxin tolerance, it would be expected to manifest itself in the passively tolerant animal as well. Such studies would avoid measuring what might be no more than concomitant changes caused by administration of endotoxin. It must be emphasized that for demonstration of passive transfer the manner in which the donors are rendered tolerant by administration of endotoxin is crucial; this is discussed elsewhere.[78] The present report describes the results of such studies on the RES of recipient animals tolerant to the effects of endotoxin by passive transfer. The hypothesis that tolerance derives from an altered state of the RES is strongly supported by these findings.

Methods

Donor rabbits. Animals of both sexes and mixed breed, weighing 2 to 2.5 kg, were obtained from a local breeder. All rabbits were acclimatized to the stocks in which they were kept during the experiments by prior training. Tolerance was induced with the lipopolysaccharide of *Salmonella typhosa* O 901 (Difco) by 6 daily doses of 2.5, 2.5, 5, 5, 10, and 10 µg I.V., and blood was taken for serum or plasma on the seventh day, 20 to 24 hours after the last dose of endotoxin. Serum or plasma from normal rabbits of the same stock was used for control. For serum, the blood was allowed to clot at room temperature and then refrigerated overnight, and the serum was collected the following day. For plasma, 1 vol. of 3.8 per cent sodium citrate was used for 5 vol. of whole blood and the plasma was collected by centrifugation the same day. Plasma or serum was vialed in single dose volumes and frozen until used.

RES blocked donor rabbits. These animals were rendered tolerant by the endotoxin treatment outlined above, but 20 hours after the last dose of endotoxin they were given I.V. 10 ml. of a carbon suspension (Gunther Wagner C 11/1431a) containing 45 mg./ml. in 2 per cent gelatin. When the blood was clear of carbon about 4 hours later, the loss of tolerance produced by the

RES blockade was confirmed in 1 animal of each group so treated by measuring the febrile response to 2.5 μg of endotoxin. These controls were exquisitely sensitive to endotoxin, their fevers exceeding both that recorded the preceding day following a dose of 10 μg of endotoxin and that found on the first day of treatment with the 2.5 μg test dose. At the same time the other rabbits were bled for serum or plasma.

Test rabbits. Normal or tolerant donor serum or plasma was administered I.V. in 10-ml vol 30 min before a test dose of 2.5 μg of endotoxin. Three hours later, the time of maximal fever in the control groups, carbon clearance rates were determined by using a carbon dose of 5 mg/100 gm body weight.

Test mice. Animals weighing approximately 18 gm were obtained from a local breeder. Serum or plasma was given I.P. in 1 ml vol. For experiments on protection against lethality of endotoxin by passive transfer an LD_{75} dose (0.6 mg I.P.) of the *S. typhosa* O 901 endotoxin was given 3 hours later. For carbon clearance studies the mice treated as described were tested with a carbon dose of 12 mg/100 gm body weight 1 hour after the endotoxin administration. In other experiments without endotoxin a carbon dose of 15 mg/100 gm was given to test for clearance 3 hours after giving serum or plasma, that is, at the time the endotoxin challenge would ordinarily have been given.

Apparatus. In order to avoid pyrogen contamination all glassware, needles and syringes were heated at 175 to 180° C for 2 to 3 hours before use. Samples of serum and plasma were proved nonpyrogenic in normal rabbits.

Carbon clearance. The Gunther Wagner C 11/1431a carbon suspension was prepared for intravenous administration by the method of Biozzi *et al.*[24] the precautions concerning dosage being observed. The heparinized blood samples were diluted with saline and centrifuged, and the supernatant carbon concentrations were measured by absorption at 1100 mμ. The rate of phagocytosis (phagocytic index) was determined for each animal in the manner originally described by Biozzi *et al.*[25] Mean rate of clearance and standard error of the mean were computed for each group, and significance of difference was assumed for $p < 0.01$. The curves were constructed by passing a line of the computed mean slope through the mean log concentration at the first measured point. The mean values were multiplied by 10^3 to eliminate decimal points and zeroes.

Results

In FIGURE 1 are given the rates of carbon clearance in rabbits 3 hours after 2.5 μg of endotoxin. The mean rate for 11 normal control rabbits was 79 ± 8, which fell to 27 ± 4 after endotoxin in a group of 7 animals. Groups of 7 rabbits each given 10 ml of normal or tolerant donor plasma 30 min before the endotoxin yielded rates of 39 ± 7 and 95 ± 14 respectively. Thus pretreatment with normal plasma, which does not modify the febrile response to the pyrogenic effect of the endotoxin, did not prevent the depression of carbon clearance. In contrast, the group pretreated with tolerant donor plasma cleared carbon normally after endotoxin under conditions wherein they also exhibit a tolerant febrile response to the pyrogen.[23]

When carbon clearance in mice was determined 1 hour after an I P LD ₅ of endotoxin similar results were obtained with an important exception (FIGURE 2) For this route of administration of endotoxin and at this time no significant change in clearance rate over that of untreated controls was found nor did the mice given normal donor plasma or serum I P 3 hours before the endotoxin differ from the controls The mice pretreated with tolerant donor serum or plasma however and for whom the endotoxin lethality wa

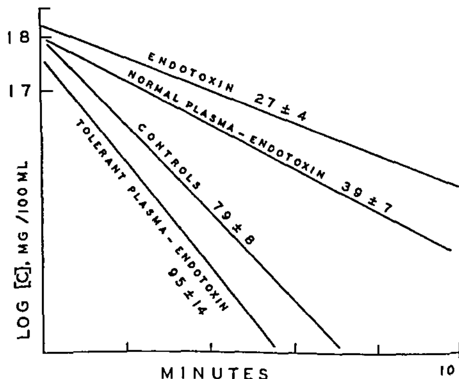


FIGURE 1 Effect of pretreatment with normal or tolerant donor plasma on endotoxin induced depression of carbon clearance rate in rabbits Carbon dose 5 mg /100 gm body weight

substantially reduced [7] displayed a carbon clearance significantly greater than the other groups including the untreated controls In this regard the mice passively tolerant to the lethality of the endotoxin resemble endotoxin tolerant animals

If a causal relationship between enhanced RES activity and endotoxin tolerance exists it would be examined best in our passively tolerant animal before endotoxin is given This was the plan of the experiments summarized in FIGURE 3 Here the rate of carbon clearance in groups of 18 to 20 mice following pretreatment with normal or tolerant donor serum or plasma given I P 3 hours earlier was determined As may be seen at the time the endotoxin challenge would ordinarily have been given the mice protected by prior

CONTROL 81 ± 11
ENDOTOXIN 86 ± 15
NORMAL & TOXIN 76 ± 27
TOLERANT & TOXIN 175 ± 22

2 2
2 0

LOG [C], MG/100 ML

MINUTES

12

FIGURE 2 Effect of passive transfer from normal or tolerant donors on carbon clearance rate in mice after an 1 1 LD_{50} dose of endotoxin Carbon dose 12 mg/100 gm body weight

CONTROL 65 ± 5
NORMAL SERUM 61 ± 10
TOLERANT SERUM 105 ± 9

2 3
2 2

LOG [C] MG/100 ML

MINUTES

12

FIGURE 3 Modification of carbon clearance rate in mice by prior administration of serum of endotoxin tolerant donors Carbon dose 15 mg/100 gm body weight

administration of tolerant donor blood cleared carbon at a significantly greater rate than did the control mice. Thus the plasma or serum of tolerant donors stimulates the RES of the normal recipient and this effect is present before the endotoxin challenge dose is given.

TABLE 1 gives data from experiments on protection against lethality of endotoxin in the mouse. Rabbits whose previously induced endotoxin tolerance was abolished by a blocking dose of carbon served as donors of plasma. Such plasma from donors no longer tolerant to endotoxin themselves still conferred protection upon normal recipients. Tolerance to the pyrogenic effect of endotoxin in the rabbit by passive transfer from these RES blocked donors has also been demonstrated.[9]

Also shown in this table is the failure of serum from tolerant donors to protect when mixed extemporaneously with the endotoxin challenge and administered in a single I.P. injection; this serum when given separately and prior to the challenge affords substantial protection against endotoxin lethality.[7]

TABLE 1

PROTECTION AGAINST LETHALITY OF LD_{75} DOSE OF ENDOTOXIN IN MICE BY PASSIVE TRANSFER FROM NO LONGER TOLERANT RES BLOCKED DONORS AND FAILURE OF TOLERANT DONOR SERUM TO PROTECT WHEN GIVEN SIMULTANEOUSLY MIXED WITH THE ENDOTOXIN

Group	No. expt	Dead/total	Dead (%)
Controls	4	27/40	68
Tolerant RES blocked plasma	2	2/20	10
Mixture tolerant serum-endotoxin	2	18/20	90

$p < 0.01$

Discussion

The serum or plasma of endotoxin tolerant donors has been shown to prevent the acute RES depression that follows intravenous endotoxin in rabbits and to produce an absolute increase in rate of clearance of carbon from the blood by the RES in mice. These effects were obtained under conditions wherein the passive transfer from tolerant donors protects against the pyrogenic[3] and lethal[7] effects of the endotoxin challenge in normal recipients. In their enhanced RES activity such passively tolerant animals resemble animals made tolerant to endotoxin by daily doses of the bacterial lipopolysaccharide. The findings strongly support the hypothesis that the state of the RES is causally related to endotoxin tolerance.

There are several implications of the results of the experiments with the no longer tolerant RES blocked donors. It would appear extremely difficult to explain endotoxin tolerance and transfer of tolerance on the basis of antibody, whether circulating or fixed. Quite apart from earlier work in this field[1] which dissociated tolerance from specific antibody, the susceptibility to endotoxin of the RES blocked donors and the ineffectiveness of the serum when

mixed with the endotoxin just before administration argue strongly against circulating antibody as the responsible agent, and an assumption of fixed antibody leaves unanswered the passive transfer of tolerance These arguments apply equally against the hypothesis of humoral inactivators of endotoxin [9] as the mechanism of tolerance

There remains the possibility that the RES-blocked donor has a protective circulating antibody but the block in some way interferes with the disposition of the hypothetical antibody-endotoxin complex however the failure to protect with the tolerant serum-endotoxin mixture would require an additional postulate Carey *et al* [30] and Ribble *et al* [31] after having made studies with labeled endotoxin have suggested that an antibody endotoxin precipitate forms instantly in the circulation of the tolerant animal The time course of events however induced them to postulate that the precipitate is not taken up by the RES but rather is trapped in the pulmonary capillaries from which it is later washed back into the circulation Furthermore Braude *et al* [7] have demonstrated in experiments with animals in which tolerance has lapsed but antibody titer is still high and with X irradiated tolerant animals lacking precipitins that circulating antibody is not essential to endotoxin tolerance The suggestion of Braude *et al* [7] that antibody allows for a temporary removal of endotoxin from the circulation that does not provide protection from the endotoxin probably is the best estimate of the situation

As to the nature of the active substance in tolerant donor blood we have eliminated certain obvious possibilities In studies to be reported separately the effects are shown to be not referable to adrenal cortical hormone properdin or the phagocytosis promoting factor of serum On the other hand we have obtained from the blood of tolerant but not normal donors a fraction that confers protection against endotoxin this also stimulates the RES in normal recipients Studies made thus far demonstrate that the active substance is a lipid The tissue source of this humoral agent which stimulates the RES of the normal animal is not known Further studies in these directions are in progress

Summary

It has been shown that passive transfer of tolerance to bacterial endotoxin may be achieved and that endotoxin tolerant donor blood stimulates the RES of normal recipient animals as determined by carbon clearance in the recipients This enhanced RES function is demonstrable before an endotoxin challenge is given The plasma of no longer tolerant RES blocked donors retains the ability to confer tolerance passively upon normal animals This and the failure to obtain protection when tolerant plasma is mixed with endotoxin and given as a single injection argue against the supposition that tolerance or its transfer is mediated by antibody to or humoral inactivator of endotoxin The experiments offer further evidence that tolerance to endotoxin is based upon an altered state in the tissues and they strongly support the hypothesis that RES function is causally related to the heightened nonspecific resistance that characterizes the endotoxin tolerant animal

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References

1 Thomas L 1954 Ann Rev Physiol 16 467
2 Bennett I L Jr & L E Cluff 1957 Pharmacol Rev 9 427
3 Zweifach B W & L Thomas 1957 J Exptl Med 106 385
4 Smiddy F G & J Fine 1957 Proc Soc Exptl Biol Med 96 558
5 Fine J E D Frank H A Ravin S H Rutenburg & F B Schweinburg 1959 New Engl J Med 260 214
6 Rowley D 1956 Brit J Exptl Pathol 37 223
7 Landy M & L Pillemer 1956 J Exptl Med 104 383
8 Boehme D & R J Dubos 1958 J Exptl Med 107 523
9 Beeson P B 1947 J Exptl Med 86 39
10 Braude A I F J Carey & M Zalesky 1955 J Clin Invest 34 858
11 Beeson P B 1947 Proc Soc Exptl Biol Med 64 146
12 Bennett I L Jr 1952 Proc Soc Exptl Biol Med 81 248
13 Zweifach B W B Benacerraf & L Thomas 1957 J Exptl Med 106 403
14 Fine J S Rutenburg & F B Schweinburg 1959 J Exptl Med 110 547
15 Good R A & L Thomas 1952 J Exptl Med 96 625
16 Smith R T L Thomas & R A Good 1953 Proc Soc Exptl Biol Med 82 712
17 Smith R T & L Thomas 1954 Proc Soc Exptl Biol Med 86 806
18 Cremer N & D W Watson 1957 Proc Soc Exptl Biol Med 95 510
19 Biozzi G B Benacerraf & B N Halpern 1955 Brit J Exptl Pathol 36 226
20 Benacerraf B & M M Sebestyen 1957 Federation Proc 16 860
21 Schweinburg F B D Davidoff I H Koven & J Fine 1956 Proc Soc Exptl Biol Med 92 662
22 Creech H J R F Haukwitz Jr & D R A Wharton 1949 Cancer Research 9 150
23 Kiser J S H Lindh & G C de Mello 1956 Ann N Y Acad Sci 66(2) 312
24 Heller J H R M Meier R Zucker & G W Mast 1957 Endocrinology 61 235
25 Biozzi G B Benacerraf & B N Halpern 1953 Brit J Exptl Pathol 34 441
26 Westphal O 1957 Pyrogens *In* Polysaccharides in Biology Trans Second Conf Josiah Macy Jr Foundation New York N Y
27 Freedman H H 1959 Proc Soc Exptl Biol Med 102 504
28 Freedman H H 1960 J Exptl Med 111 453
29 Farr R S S L Clark Jr & J E Proffitt 1954 Am J Physiol 177 269
30 Carey F J A I Braude & M Zalesky 1958 J Clin Invest 37 441
31 Ribble J C M Zalesky & A I Braude 1959 Bull Johns Hopkin Hosp 105 272
32 Braude A I F J Carey & M Zalesky 1955 J Clin Invest 34 923

EFFECT OF CORTICOSTEROIDS AND OF HORMONES OF PREGNANCY ON THE LETHAL ACTION OF BACTERIAL ENDOTOXIN*

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In only two situations have adrenocortical hormones been shown to be protective to the host the replacement of hormone in hypoadrenalism and the protective action against the lethal toxicity of bacterial lipopolysaccharides *In the first instance the dose of administered hormone is critical* For example, intact mice were given sufficient antiserum to protect them against about 50 000 pneumococci adrenalectomized mice given the same dose of antiserum were killed by as few as 1000 pneumococci (FIGURE 1) The administration of cortisone restored the resistance of the adrenalectomized mice to preoperative levels but not to level of resistance greater than were seen in intact animals When the dose of cortisone was increased beyond the level that restored optimal resistance to infection the resistance of the animals decreased once again[1]

The second protective action of corticosteroids is that manifested against the action of bacterial lipopolysaccharides In 1946 Lewis and Page[2] demonstrated that corticosteroids overcame the lethal action of typhoid vaccine in adrenalectomized animals In 1950 Beck and Voloshin[3] observed that the *tumor necrotizing effects of a bacterial lipopolysaccharide were attenuated* if the tumor bearing animals had previously received cortisone or adrenal cortical extract Also in 1950 it was observed in our laboratory and by Recant *et al*[4,5] that pretreatment with corticotropin or with cortisone diminished the febrile response to pyrogens in man and in rabbits Shortly thereafter Chedid *et al*[6] observed that cortisone protected the intact animal against the lethal action of bacterial endotoxins and the finding has been amply confirmed

Subsequent studies have shown that cortisone protects mice rats rabbits and chick embryos against the simultaneous or subsequent injection of lethal doses of bacterial lipopolysaccharide[1,7,8,9,10] Guinea pigs are not protected against the lethal action of endotoxins by pretreatment with cortisone and probably dogs are likewise not protected[11,12] The protective effect of corticosteroids is apparently dependent upon the presence of increased blood levels of corticosteroids at the time endotoxin is administered A remarkable influence of age of the host on the response to bacterial lipopolysaccharides was observed by Thomas and Smith[13] Large rabbits weighing 2 to 4 kg were protected against the lethal action of endotoxin by the prior administration of corticosteroid However when smaller rabbits (<1 5 kg) were protected with corticosteroids and then given endotoxin bilateral renal cortical necrosis often resulted The initial dose of cortisone in the small rabbits supplanted the preparatory dose of endotoxin in eliciting the generalized Shwartzman phenomenon

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In contrast to the effect of corticosteroids on resistance to infection the protective effect of these hormones against the lethal action of endotoxin is not biphasic, that is large doses of cortisone do not increase susceptibility to the toxic lipopolysaccharides On the other hand adrenalectomy increases susceptibility to the lethal action of endotoxins by approximately one thousandfold, and replacement of the hormone affords protection as demonstrated above

The protective action of corticosteroids on adrenalectomized animals provides a satisfactory assay for measuring antiendotoxic action [2, 14] Thus if an LD_{50} dose of endotoxin is given intracardially to adrenalectomized rats weighing 100 to 150 gm, as little as 10 μg of cortisol provides measurable protection [14]

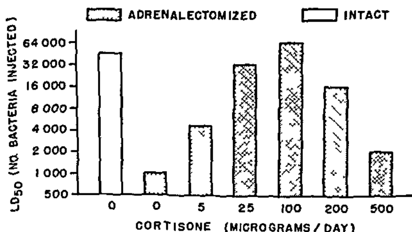


FIGURE 1 Dose sufficient to protect 50 per cent of intact mice against 50 000 virulent pneumococci Adrenalectomy markedly enhanced susceptibility to pneumococcal infection as shown by the much lower LD_{50} Cortisone (5 or 25 μg /day) partially restored resistance of adrenalectomized mice whereas dose of 100 μg increased LD essentially to that of control mice Larger doses of cortisone are necessary to produce fatal infection

Similar assays dependent upon protection against other aspects of the toxic response to endotoxin have been developed [9, 15, 16]

The hormone may be mixed with endotoxin and the mixture given intracardially The methods using such a mixture are sensitive and the end points are definitive Within a tenfold range of steroid concentration the response to the corticosteroid is linear In addition the intracardiac injection allows measurement of biological activity of the corticosteroids in a system in which turnover from a tissue site of deposition is not a critical feature of the assay

The factor of turnover from tissue sites of deposition has received insufficient attention It can be shown readily for example that cortisol and corticosterone are equally protective against bacterial endotoxin when these are given intravenously (TABLE 1) The differences in the action of these hormones after

intramuscular or subcutaneous injection are due to differences in the rates of absorption of the hormones from the tissue sites of deposition [8]

Since virtually all of the comparative biological observations similar to those presented in this symposium have been made with corticosteroids injected into local tissue sites, the question may be raised whether biologically active materials have been overlooked simply because they happen to be mobilized more rapidly than standard reference steroids In addition the usual studies which involve subcutaneous or intramuscular administration of steroid may not have given us an accurate view of the relationship of steroid structure to biological function Indeed in most studies in which corticosteroid have been administered subcutaneously or intramuscularly cortisol has been shown to have actions strikingly different from corticosterone [17, 18] It may fairly be asked whether this observation is not in part at least an artifact of the method of administration of the hormone

TABLE 1
ROUTE OF ADMINISTRATION AND PROTECTION BY CORTISOL AND CORTICOSTERONE AGAINST LETHAL ACTION OF ENDOTOXIN IN INTACT RATS

Route and dose	Interval between hormone and toxin (hours)	Cortisol treated	Corticosterone treated	Control
Subcutaneous 10 mg	24	5/8	0/8	1/8
	8	6/8	3/8	0/8
	2	8/8	6/8	3/8
	1	6/8	5/8	1/8
Intraperitoneal 10 mg	8	2/8	[illegible]/8	1/8
	0 5	8/8	6/8	1/8
	~1	3/8	2/8	1/8
Intracardiac 0 5 mg	[illegible]	3/8	1/8	3/8
	1	5/16	2/16	5/16
	0	1[illegible]/16	12/16	3/16

Number surviving per total number injected Adapted from Levitin *et al* [9]

By taking advantage of the assay systems that remove the factor of turnover from local sites of deposition new relationships between structure and function may be uncovered For example the antiendotoxic activity of corticosteroids seems to be sufficiently different from the anti inflammatory activity to offer the possibility that these two activities may be structurally separable The anti inflammatory action of corticosteroids is apparently related largely to an effect on vascular permeability whereas the antiendotoxic activity in some way involves the reticuloendothelial system It is noteworthy also that substances such as chlorpromazine serotonin and dibenamine also protect experimental animals against the action of endotoxins [19, 20] presumably through mechanisms of action that are not anti inflammatory

When the relative antiendotoxic activities of corticosteroid are determined using the intracardiac route cortisol proves to be slightly more active than is cortisone which in turn is about as active as corticosterone (TABLE 2) Deoxy

corticosterone 17 OH progesterone, and progesterone are quite inactive. Interestingly 6-methyl prednisolone is about 30 times as active as cortisol in this assay but is only about 5 times as active as cortisol as a glucocorticoid. Glucocorticoid activity of course, has generally correlated well with antiinflammatory activity.

Specifically antiendotoxic substances would be valuable not only as tools in *the study of endotoxin and the reticuloendothelial system* but also because there might be therapeutic value to the use of such substances in Gram negative rod infections, many of which are associated with a disconcertingly high mortality rate.[1]

The mechanisms by which corticosteroids interfere with the lethal action of endotoxins is unknown. In a search for possible approaches to the mechanism of action of corticosteroids the evidence that there were specific combining sites for endotoxin in different tissues and perhaps at the level of cell membranes was reviewed. The studies of Westphal and his associates[22, 23] have demonstrated the occurrence in bacterial lipopolysaccharide of unusual hexoses

TABLE 2

RELATIVE ANTI INFLAMMATORY (GLUCOCORTICOID) AND ANTIENDOTOXIC EFFECTS OF STEROIDS

Steroid	Antiinflammatory	Antiendotoxic
Cortisol	1.0	1.0
Corticosterone	0.5	0.7
Prednisone	3.5	0.7
Prednisolone	4.0	1.3
6-α Methyl prednisone	5.0	30.0
11 Deoxycorticosterone	<0.1	0.1
11 α OH progesteron	<0.1	0.03

deoxygenated at two different carbons on the chain. At present no evidence of the occurrence of such hexoses in mammalian tissues has been obtained. Furthermore no direct evidence of a combining site for endotoxin has been adduced in any of the experiments so far reviewed. However inferential evidence of a chemical similarity between bacterial lipopolysaccharides and substances in mammalian tissue may be derived from the demonstrations that the blood from normal animals alters bacterial lipopolysaccharides.

The evidence for such activity is based upon the finding that incubation of bacterial lipopolysaccharides with serum (1) alters the antigenic behavior *in vitro* of lipopolysaccharides,[4, 5] (2) causes radioactive phosphate to be split from purified lipopolysaccharide into which the phosphorus had been incorporated,[26] (3) attenuates the pyrogenic activity of lipopolysaccharides,[7, 9] although in some instances augmentation of the pyrogenic response may be observed for a brief time following such incubation,[4, 5, 31] and (4) attenuates the lethal and tumor necrotizing effects of bacterial endotoxin.[33] The hypothesis that all or most of the attenuating activities are due to the same substance in serum seems to be a reasonable one. The serum substance may possibly be a phosphatase but is different from the serum alkaline phosphatase

It occurs in Cohn Fraction II + III but not in the gamma globulin fraction It migrates with the beta globulins It is inhibited by exposure to mercuric or arsenical salts and i therefore perhap a sulfhydryl-dependent enzyme Its re ponse to heat suggests an enzymic effect (FIGURE 2) Its activity is uch that as little as 0 025 ml of Fraction II + III that had been reconstituted to the concentration found in the original pla ma reduced the lethal toxicity of endotoxin significantly [7]

The process of extracting the lipopolysaccharide that is the sub trate for thi enzyme i critical to an adequate demon tration of its activity The lethal toxicity of lipopolysaccharides prepared by trichloroacetic acid extraction was not affected by the serum enzyme whereas lipopolysaccharides prepared from

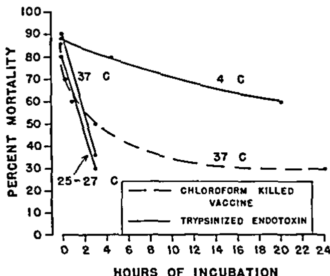


FIGURE 2 Effect of temperature and duration of incubation on attenuation of lethal ac tion of endotoxin

chloroform killed phenol killed or heat killed bacterial suspensions were detoxified by the serum factor From the studies of Westphal and his associates it is clear that the lipid B fraction of bacterial lipopolysaccharide is readily hydrolyzed yet its loss does not alter the lethal toxicity or pyrogenicity of endotoxin It may be suggested therefore that the lipid B portion i one of the specific sites of action of the serum factor on endotoxin as a possible way of harmonizing the finding that trichloroacetic acid extraction does not alter lethal toxicity but does alter the capacity of endotoxin to be affected by the serum enzyme The activity of the serum factor is separable from anti body properdin and complement [7]

The finding of this activity in serum from rats rabbits and man and the finding of apparent substrates in mou e tumors erythrocytes and a plant suggest that the substrate for the enzyme or chemically similar substrates have

wide biological distribution Further study of their distribution may give some insight into the action of lipopolysaccharides Perhaps indirectly further information concerning the mode of action of corticosteroids will also be obtained Certainly, the need for continued search for substances similar to bacterial lipopolysaccharides in mammalian tissues and the need for confirming the observations of Shear and Landy[33] on the biological distribution of endotoxin like substances is great

It is obvious that the foregoing discussion is largely speculative and of limited value beyond providing a matrix for further investigation Many discrepancies are already apparent such as differences in observed heat stability of the serum factor in different study systems and differences in the apparent requirements for divalent cations in different systems The elucidation of the nature of the serum factor and of its mammalian substrate is a problem of some biological interest and its implications are broad

Recent observations in our laboratory along somewhat different lines have also uncovered an aspect of the problem that has broad biological implications We have been interested in our laboratory in the study of certain chronic infections that occur in an asymptomatic form in man Pyelonephritis is an example of this type of infection Our initial approach was to determine the methods for the detection of asymptomatic forms of infection of the urinary tract By quantitative study of the bacterial flora of freshly voided urine it has been possible to detect a large reservoir of asymptomatic infections of the urinary tract[34-36] The finding of more than 100 000 bacteria (usually Gram negative rods) per milliliter of voided urine suggests with reasonable assurance that the individual from whom the specimen was obtained has asymptomatic by persistent bacteriuria and a substantial but as yet incompletely plotted, likelihood of having pyelonephritis[37,38]

Recently the urines of more than 4000 women making their first prenatal visits to the outpatient department of the Boston City Hospital were studied The incidence of bacteriuria was found to be 6 to 7 per cent in this group, and in order to document the predictive value of bacteriuria a simple controlled therapeutic study was undertaken Patients who were less than 8 months pregnant with bacteriuria documented on at least 2 separate occasions were divided by alternation into 2 groups Members of one group were treated to eliminate the bacteriuria and those in the other group were given a placebo Extended clinical study of these patients showed that about 40 per cent of pregnant women with bacteriuria were destined to develop pyelonephritis during pregnancy or during the immediate post partum period No woman whose bacteriuria was eliminated throughout pregnancy developed pyelonephritis of pregnancy Thus pyelonephritis of pregnancy is apparently more or less completely preventable, and bacteriuria has predictive value in pregnancy[38]

When the fates of the infants that were born to the bacteriuric mothers were studied an unusually high incidence of prematurity and of infant mortality in untreated bacteriuric mothers was found (TABLE 3) The incidence of prematurity in untreated bacteriuric mothers is about 25 per cent The incidence of perinatal death in the bacteriuric women is 14 per cent[39]

Patients who were kept free of bacteriuria up to the time of delivery had an

incidence of prematurity of 9 per cent and there were no neonatal deaths in this small group The incidence of prematurity in 1000 consecutive nonbacteriuric mothers was 9 per cent in this clinic If the implication of these data are borne out in further studies that are now in progress it may be possible to avoid 10 to 20 per cent of prematures and approximately 25 to 30 per cent of perinatal deaths by detecting and treating bacteriuria during pregnancy

How do these findings relate to endotoxin and its activity? Autopsy studies of the dead babies and clinical observations of the mothers and babies offered no indication that active infection had spread from the urinary tract to precipitate labor and produce sepsis It has been known for many years however that the pregnant rabbit reacts to a single dose of endotoxin with the production of a generalized Shwartzman reaction and that endotoxin often produces placental necrosis and abortion[40, 41] Studies by Samir N Hajj Lauri D Thrupp Ennio Vivaldi and Donald P Zangwill in our laboratory have shown that when pregnant rabbits are given endotoxin sustained and severe uterine contractions occur within less than 1 min The nonpregnant uterus

TABLE 3

EFFECT OF BACTERIURIA DURING PREGNANCY ON OCCURRENCE OF PYELONEPHRITIS PREMATURITY AND PERINATAL DEATH

Patient group	No. of patients	No. with pyelonephritis	Premature infants (%)	Perinatal mortality
Untreated bacteriuric	48	20	24	14
Treated bacteriuric	43	0	10	0
Nonbacteriuric	1000	0	9	2

reacts very little to injections of endotoxin nor does other smooth muscle from the pregnant animal In some manner then the hormonal changes of pregnancy appear to sensitize the uterus to rapid response to endotoxin So striking is this sensitization that it is occasionally possible to demonstrate *in vitro* in a water bath that strips of uterus taken from the pregnant rabbit near term will respond to the addition of endotoxin to the bath *

Several aspects of this response of the pregnant uterus to endotoxin are noteworthy First there is a very brief lag period in contrast to the usual pattern of response to the administration of endotoxin The uterine contractions occur within one or two minutes after endotoxin has been administered Second all indications are that the hormones of pregnancy in some way sensitize the uterus to the action of endotoxin Third the patients in the series show findings consistent with the latter observation Those patients who became ill and were freed of bacteriuria by treatment did not have an increased incidence of perinatal death and of bacteriuria This would suggest then that the persistence of bacteriuria late in pregnancy is the critical feature in the development of prematurity and that the elimination of bacteriuria consequent

* Recently Thiersch has observed that bacterial lipopolysaccharides in small doses induce fetal death and stunting in rats and that the effect of the lipopolysaccharides was most severe after mid term

to the production of clinical illnes was the key feature in the lower incidence of prematurity in those patients who became ill The situation pre ented by the occurrence of bacteriuria in pregnancy would appear to be a clinical re flection of an influence of endotoxin and the mechanism by which this occurs obviously requires further studies

At present a unifying hypothe is may be suggested If it be assumed that *endotoxin is attracted by specific combining sites at cell membrane levels and* that the rate of entry of endotoxin into these cells determines its lethal action it could be suggested that adrenal steroids slow the rate of entry of endotoxin into critical cells by their action on cell membranes and conversely hormones of pregnancy accelerate the rate of entry thus accounting for the absence of a lag period Endotoxin therefore might be expected to permit the entry of many substances by influencing permeability mechanisms It may be sug gested for example that it would allow oxytocin to act more readily, and pre liminary experiments by Hajj in our laboratory suggest that the uterus that has been stimulated by endotoxin is much more responsive to oxytocin than otherwise

It is apparent that new dimensions to the problem of the physiological action of endotoxin are appearing and that these are beginning to take a form that suggests that certain problems of disease in man will be understood better by further study of the action of endotoxin

References

1 Kass E H & M Finland 19 8 Corticosteroids and infection Advances in Intern Med 9 45

2 Lewis L A & I H Page 1947 Methods of assaying teroids and adrenal extracts for protective action against toxic material (typhoid vaccine) J Lab Clin Med **31** 1375

3 Beck L V & T Voloshin *1950 Influence of adrenal hormones on toxic and tumor* damaging effects of certain substances Am J Physiol **163** 696

4 Kass E H & M Finland 19 0 Effect of ACTH on induced fever New Engl J Med **243** 693

5 Recant L W H Otto & E E Fischel 1950 Antipyretic effect of cortisone Proc. Soc Exptl Biol Med **75** 264

6 Chedid L F Boyer & M Saviard 1954 Action de la cortisone vis à vis de l infec tion experimental avec *Salm mella typl i* chez la souris Compt rend acad sci **233** 713

7 Geller P E R Merrill & E Jawetz 1954 Effect of corti one and antibiotics on lethal action of endotoxins in mice Proc Soc Exptl Biol Med **86** 716

8 Levitin H M I Kendrick & E H Kass 19 6 Effect of r ute f administration on protective action of corticoster ne and c tisol against en lot xin Proc Soc Exptl Biol Med **93** 306

9 Smith R T & L Thomas 1956 Lethal effe t of endot xins n chick embryo J Exptl Med **104** 217

10 Spink W W & D Anderson 1954 Expe imental tu lies on sign f cance of endo toxin on pathogenesis of brucellosis J Clin In est **33** 540

11 Borden C W W H Hall & D Gold 1953 St ly f xperiment l hock produced by meningococcus toxin J Lab Clin Med **42** 787

12 Chedid L F Boyer & M Saviard 19 4 N u eaux spects de la f nction anti toxique de la cortisone Ann inst P teur 86 34

13 Thomas L & R T Smith 1954 Effect f cortis e on ponse to en l toxin in ma ture rabbits Proc Soc Exptl B ol Med 86 810

14 Brooke M E H Kass & O Hechter 1959 Prote ti e effect of steroi l against bacterial endotoxin Federation Proc 18 560

15 Hormone Assay 1950 375 E W Emmens Ed Aca lemic Press New York N Y

16 Gass G H & E J Umberger 1959 Sur i al time f endotoxin treated rats as re

ponse metameter for corticotropin and hydrocortisone bioassays Proc Soc Exptl Biol Med 100 127
17 Kendall, E C 1949 Relation of chemical structure of adrenal cortical hormones to biological activity Trans First Conf on Adrenal Cortex 11 Josiah Macy Jr Foundation New York N Y
18 Kass E H *et al* 1955 Effects of adrenal steroids on resistance to infection differences in relative amounts of corticosterone and hydrocortisone secreted and in their biologic effects A M A Arch Internal Med 96 397
19 Reilly J & I Tournier 1953 Action de la chlorpromazine (4560 RP) sur l in toxication typhique experimentale Bull acad natl med Paris France 137 385
20 Abernathy R S F Halberg & W W Spink 1957 Studies on mechanism of chlorpromazine protection against brucella endotoxin in mice J Lab Clin Med 49 708
21 Finland M W F Jones & M W Barnes 1959 Changes in incidence of serious acute bacterial infections since introduction of antibacterial agents Trans Assoc Am Physicians 77 305
22 Westphal O 1952 Bakterienreizstoffe und ihre Wirkungsweise Angew Chem 64 314
23 Fromme I K Himmelspach O Lüderitz & O Westphal 1957 Analyse der Zuckerbausteine in Bakteriellen Lipopolysacchariden Angew Chem 69 643
24 Cluff L E 1956 Study of effect of serum on immunological reaction of bacterial endotoxin J Exptl Med 103 439
25 Stalnch J F & A C Johnson 1957 Alteration of bacterial endotoxins by human and rabbit serum Federation Proc 16 434
26 Rowley D 1956 Some factors affecting the resistance of animals to bacterial infection Ann N Y Acad Sci 66(2) 304
27 Goodale F Jr E S Snell F Wendt & W I Cranston 1956 Inactivation of bacterial pyrogen by human serum and plasma Clin Sci 15 491
28 Hegemann F 1954 Zur Bedeutung des Blutserums für die Entstehung und das Unwirksamwerden bakterieller Reizstoffe beim Menschen he humorale Entstehung entzündungs und fiebererregender Stoffe aus Colibakterien Z Immunitätsforsch 111 207
29 Farr R S & A S LeQuire 1951 Leukocytic and pyrogenic effects of typhoid vaccine and augmentation by homologous plasma Proc Soc Exptl Biol Med 75 661
30 Grant R & W J Whalen 1953 Latency of pyrogen fever Appearance of fast acting pyrogen in blood of febrile animals and in plasma incubated with bacterial pyrogen Am J Phys ol 173 47
31 LeQuire V S 1951 Augmentation of thermogenic effects of pyrogens by homologous plasma in rabbits J Infectious Diseases 88 194
32 Ho M & E H Kass 1958 Protective effect of components of normal blood against the lethal action of endotoxin J Lab Clin Med 51 297
33 Landy M R C Skarnes F S Rosen R J Trapani & M J Shear 1957 Inactivity of biologically active endotoxic polysaccharides by fresh human serum Proc Soc Exptl Biol Med 96 744
34 Kass E H 1955 Chemotherapeutic and antibiotic drugs in the management of infections of the urinary tract Am J Med 18 764
35 Kass E H 1956 Asymptomatic infections of the urinary tract Trans Assoc Am Physicians 69 56
36 Kass E H 1957 Bacteriuria and the diagnosis of infections of the urinary tract with observations on the use of methionine as a urinary antiseptic A M A Arch Internal Med 100 709
37 MacDonald R A H Levitin G K Mallory & E H Kass 1957 Relation between pyelonephritis and bacterial counts in the urine An autopsy study New Engl J Med 256 915
38 Kass E H 1959 Bacteriuria and pyelonephritis of pregnancy Trans Assoc Am Physicians 72 257
39 Kass E H Hormones and host resistance to infection Bacteriol Rev In press
40 Zahl P A & C Bjerknes 1943 Induction of decidua placental hemorrhage in mice by endotoxins of certain Gram negative bacteria Proc Soc Exptl Biol Med 54 329
41 Rieder R F & L Thomas 1960 Studies on the mechanisms involved in the production of abortion by endotoxin J Immunol 84 189
42 Thiersch J B 1960 Effect of lipopolysaccharides of gram negative bacilli on the rat litter in utero Proc Soc Exptl Biol Med In press

NONTOXIC RES STIMULATORY LIPIDS*

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Over the course of the last six years several members of our laboratory have been involved in the study of various microbial fractions that might have a stimulatory effect upon the RES. As early as 1953 my associates and I suspected that such fractions existed and that they were essentially lipoid in nature as opposed to a lipoprotein or lipopolysaccharide. Initially we began to work with pathogenic microorganisms as did many others in laboratories here and abroad. It was quite feasible to extract lipopolysaccharides from these pathogens which were stimulatory but toxic, as well as some polysaccharide free lipids which were also toxic.

The results we achieved were very similar to those obtained in other laboratories. Because of the constant problem of toxicity and because we were interested in nontoxic microbial moieties, we shifted our attention from pathogenic microorganisms to nonpathogens.

One of the earliest substances used in this study was zymosan. As I mentioned in an earlier monograph published by The New York Academy of Sciences in 1958,[1] we chose zymosan for the reason that much of the data adduced by investigators working with the properdin mechanism was necessarily mangled rather badly in the attempt to make it fit a presumptive critical role of properdin in nonspecific resistance. If one did not torture the data, the numbers obtained gave such classic RES curves that association of zymosan and RES stimulation was inevitable.

Any preparation of zymosan is a potpourri of lipids, polysaccharides, proteins, and ash of variable concentration. Furthermore, there are many different varieties of zymosan that obtain as a function of the preparative procedures used. Although our laboratory initially used the term zymosan, we have decided not to utilize it any longer. If all yeast cell wall preparations were called zymosan, it would be quite confusing, as many such products which are active in the properdin reaction have no ostensible effect upon the RES. Other fractions or zymosans do have a stimulatory effect. For want of an other term we shall use for convenience the term restim to indicate a microbially derived nonpyrexic, nontoxic cellular moiety. Restim is obviously a contraction of RES stimulant.

Whereas initially we began our investigation with *Saccharomyces cerevisiae* and a standard preparation of yeast cell walls derived from this organism, we have explored thus far 38 cell wall fractions. Many of them are completely inert or provide a simple mechanical blockade of the RES followed by a mild and expected compensatory stimulation that would obtain from any relatively inert colloid.

All of these data with the different types of procedures used are too voluminous to be dealt with here and will be presented elsewhere. Suffice it to say

* The work described in this paper was supported in part by grants from the Office of Naval Research and the National Science Foundation, Washington, D. C.

that since most of the RES active lipid is apparently lodged between two polysaccharide layers of the cell wall and since the lipid is relatively fragile in a chemical sense, it requires a protracted and somewhat tedious procedure beginning with the whole cell and ending with the final lipid material.

Regrettably we cannot characterize the lipid specifically at the present time, much less identify it chemically. The lipid product that we are using currently is still a mixture, although it is free of all polysaccharide and protein. Thus far we have gone through just more than 100 fractions obtained by various physical and organic procedures. We believe that we are approaching that phase of the work where we can hope soon to characterize and then specifically identify the lipid material.

V. Z. Pasternak of our laboratory has been carrying out this fractionation procedure. The activity of the cell extracts is determined first by the effect of the lipid on phagocytosis and second on other host defense mechanisms including antibody formation.

J. L. Cutler of our laboratory has done extensive work on the role of restim in the production of antibodies, using the production of hemolysins as an index,[2] while D. A. Boroff has studied antibodies to soluble antigens. These data along with those of M. Kojima of our laboratory, which are also presented in this monograph, have persuaded us that the lipid is essentially the active component of the cell wall fraction (FIGURE 1). Therefore we have not attempted to reproduce the antibody studies with more than 100 different lipid fractions but have concentrated only on the most active; indeed, for the final definitive studies of the effect of lipid on the entire host defense mechanism, we shall wait until we have a chemically isolated entity.

The many specific chemical fractionation procedures undertaken and the measures that must be taken to protect the lipid are again too voluminous for inclusion here and will be presented elsewhere.

Along with the chemical data for later presentation is the methodology needed to put these various lipids into an adequate emulsion for intravenous injection. It should be mentioned here, however, that one of the dangers and fallacies in some of the earlier work with zymosan and other particulates relates to particle size. The injection of any intravenous particulates that have a single dimension greater than 4 μ presents a serious possibility of providing multiple microemboli in the lungs. Restim must be specially ground under conditions where excessive heating does not obtain and oxygen and moisture are excluded in order to produce particulates whose greatest diameter is less than 4 μ. These particles then must be made into a good suspension maintaining its colloidal dispersion not only in water, saline, or glucose, but actually in plasma. If tiny particulates aggregate because of the physicochemical changes that occur on their surfaces in the blood, all the careful preparative work has been done in vain. The same thing is true, of course, of lipid emulsions intended to be injected intravenously.

In the experiments to be reported here, all materials were injected intravenously either in the tail vein of the mouse or the saphenous vein of the rat. Most of the results were obtained by study on CFN white rats of varying ages and sizes, although in each group the sizes were maintained constant in the experimentals and controls. Only male rats were used.

NONTOXIC RES STIMULATORY LIPIDS*

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Over the course of the last six years several members of our laboratory have been involved in the study of various microbial fractions that might have a stimulatory effect upon the RES As early as 1953 my associates and I suspected that such fractions existed and that they were essentially lipoid in nature as opposed to a lipoprotein or lipopolysaccharide Initially we began to work with pathogenic microorganisms as did many others in laboratories here and abroad It was quite feasible to extract lipopolysaccharides from these pathogens which were stimulatory but toxic as well as some polysaccharide free lipids which were also toxic

The results we achieved were very similar to those obtained in other laboratories Because of the constant problem of toxicity and because we were interested in nontoxic microbial moieties we shifted our attention from pathogenic microorganisms to nonpathogens

One of the earliest substances used in this study was zymosan As I mentioned in an earlier monograph published by The New York Academy of Sciences in 1958[1] we chose zymosan for the reason that much of the data adduced by investigators working with the properdin mechanism was necessarily mangled rather badly in the attempt to make it fit a presumptive critical role of properdin in nonspecific resistance If one did not torture the data the numbers obtained gave such classic RES curves that association of zymosan and RES stimulation was inevitable

Any preparation of zymosan is a potpourri of lipids polysaccharides proteins and ash of variable concentration Furthermore there are many different varieties of zymosan that obtain as a function of the preparative procedures used Although our laboratory initially used the term zymosan we have decided not to utilize it any longer If all yeast cell wall preparations were called zymosan it would be quite confusing as many such products which are active in the properdin reaction have no ostensible effect upon the RES Other fractions or zymosans do have a stimulatory effect For want of another term we shall use for convenience the term restim to indicate a microbially derived nonpyrexic nontoxic cellular moiety Restim is obviously a contraction of RES stimulant

Whereas initially we began our investigation with *Saccharomyces cerevisiae* and a standard preparation of yeast cell walls derived from this organism we have explored thus far 38 cell wall fractions Many of them are completely inert or provide a simple mechanical blockade of the RES followed by a mild and expected compensatory stimulation that would obtain from any relatively inert colloid

All of these data with the different types of procedure used are too voluminous to be dealt with here and will be presented elsewhere Suffice it to say

* The work described in this paper was supported in part by grants from the Office of Naval Research and the National Science Foundation Washington D C

trol usually varied from a five fold increase to an increment of three orders of magnitude depending upon the system used

Thus in the studies done in our laboratory using particulate antigens, it could be demonstrated that antibody stimulation occurs whether restim has been injected prior to the antigen or at the same time as the antigen or up to two days after the antigen as seen in TABLE 1

These data differ in two notable characteristics from information adduced by others who have studied endotoxins or lipopolysaccharides Two of the most constant findings with endotoxins have been (1) that they are toxic and can produce pyrexia[5] and (2) that they cannot stimulate antibody production if they are given prior to the antigen[6] These two findings have led previous authors to the conclusion that it was the innate toxicity and/or pyrexia produced properties of the lipopolysaccharides that were responsible for stimulating host resistance They further ventured to guess that unless a stress reaction was produced one could not hope to obtain an increase in nonspecific resistance

TABLE 1

RESTIM INJECTED INTRAVENOUSLY IN CFN MALE RATS

[illegible] (days)	[illegible] P = <0.01
−30	+1470
−2	+310
0	+340
+2	+365

We have made rectal thermistor temperature measurements in animals at various periods after the intravenous administration of active lipid fractions and measurements taken from 10 min to 72 hours later showed no pyrexia We have seen no gross manifestations of toxicity or inability to tolerate relatively massive doses of restim (30 mg kg I V) or the active stimulatory lipids derived therefrom It is certainly true that some of the lipids that can be obtained from microbial cell walls are toxic However those we are utilizing do not appear to have this toxicity

In addition to particulate antigens we have also explored some soluble ones We do not as yet have as many data on soluble antigens as we do on particulate antigens One of the soluble antigens that has been studied is botulinum toxoid Normally the first injection of botulinum toxoid in mice is followed by a second one given 20 days later A challenge of botulinum toxin is given at 40 days and the poor survival rate indicates that the toxoid is a relatively poor antigen If the initial dose of toxoid was accompanied with either restim or the lipid material the increment in immunity at the time of challenge rose from 10 to 100 times greater than that seen in the controls which had been given only toxoid (TABLE 2)

Again with the soluble antigens it can be demonstrated that there is an

Restim produces a marked stimulation of phagocytosis, as well as a significant increment in antibody production It must be emphasized that phagocytosis can be a discrete function and can be stimulated without a concomitant

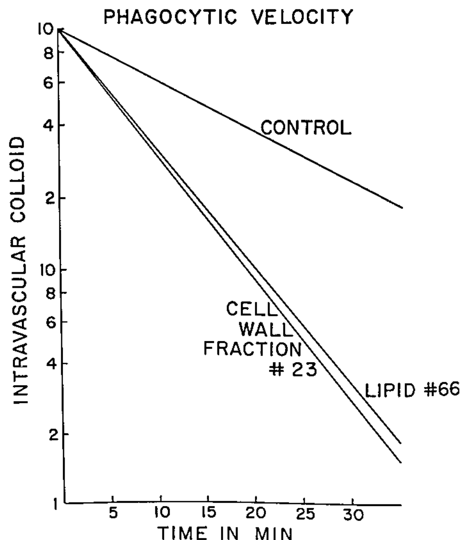


FIGURE 1 The increment of phagocytosis due to restim (cell wall fraction No 23) and the lipid (No 66) derived from this fraction are shown in contrast to control values

stimulation in the rate or quantity of antibody production Thus we have been able to increase phagocytosis significantly with synthetic estrogens but no protective effect against microbial infection was obtained as a function of this increment[3,4] However in the case of restim phagocytosis and increased antibody production go together The increase in antibody titers over con

restim and the stimulatory lipid are in all probability on a later phase in antibody production

Useful speculation on this subject must include consideration of the interesting morphologic changes that are involved as a result of this type of RES stimulation These data will be found in Mizu Kojima's presentation else where in these pages

In conclusion it may be stated that specially prepared cell walls from micro organisms can stimulate the RES as evidenced by phagocytosis and antibody production The preparation of cell walls must be carefully carried out or activity is lost The active component appears to be a lipid or a mixture of lipid obtained from the cell walls Chemical fractionation has eliminated many lipids normally occurring in these cell wall that are inert or toxic The polysaccharide protein ash residues are not active

References

1 Heller J H 19 9 Measurement of the function of the reticuloendothel sm Ann N Y Acad Sci 73(1) 212
2 Cutler J I 1960 The enhancement of hem ly in production in the rat by zym osan J Immunol 84
3 Heller J H R M Meier R A Zu ker & G W Mast 1957 The effect of natural and synthetic estrogens on reticul endothelial system function Endocrin logy 61 235
4 Heller J H & R M Meier 1960 Effect of certain synthetic steroids on rabbit hypercholester lemia RFS Bull In press
5 Johnson A G S Gaines & M Landy 1956 J Exptl Med 103 823
6 Kind P & A C Johnson 1957 J Immunol 82 415
7 Juhlin L 1960 Effects of starvation on the reticuloendothelial system as evaluated by means of a new fluorescent particle technique *In* Reticuloendothelial Structure and Function Ronald New York N Y

increment in antibody titer whether the restim is given prior to at the same time as, or after the antigen

It is intriguing to speculate on mechanisms involved in the stimulation of RES activity We can demonstrate histologically that restim and the lipids are taken up from the circulation by reticuloendothelial cells We can demonstrate also that major stimulation is accompanied by proliferation of reticuloendothelial elements A convenient assumption in terms of familiar mechanisms would include phagocytosis irritation and proliferation Thu there would simply be more reticuloendothelial cells and hence the RES would be stimulated' However such a simplified explanation does not stand up under close scrutiny First there is considerable functional stimulation 24 hours after restim administration at a time when morphologic evidence of proliferation is minimal Next although the RES stimulus from a single injection of restim lasts for some time—antibody response is m iny times normal for six weeks—the RES response returns to normal after this period even though hyperpl isia induced by the restim remains far longer Hyperphagocytosis as a result of an increased number of cells is a simple concept

TABLE 2

	C t ol	R t m	T d l e	R t m a d t o d g n t m tim	R tim g 72 h p t t o d	R stim g 4 hou ft t xo d
LD 0 as ml of toxin	2×10^{-5}†	10^{-5}†	$0\ 5 \times 10^{-5}$†	10^{-4} to 10^{-3}†	10^{-2}†	10^{-2}†

Repeated 20 days later Toxin challenge given at fortieth day
† Ten mice for each point $P = <0\ 01$

It is far more complex to try to postulate a mechanism for increased rate of phagocytosis in a single cell since we do not understand the biophysical ba is underlying phagocytosis in the first place The basic postulate that a reticuloendothelial cell could identify an electronegative surface charge on a particle has recently been challenged by data that indicate that a net electropositive charge works equally well It is completely speculative as to how the preliminary simultaneous or later phagocytosis of restim or the lipid stimulates replication or mobilization of reticuloendothelial cells or hyperactivity of extant cells It is a vital speculation however particularly in view of the observed effect on antibody synthesis A first approximation regarding this latter phenomenon might be predicated on the basis that more antigen was phagocytosed in reticuloendothelial stimulated animals than the controls However certain radioactively tagged particulate antigens that normally disappear from the circulation in a very few minutes can be shown by microradioautography to be totally phagocytosed by the RES

It is difficult to believe that there could be any differences in ultimate antibody formation as a function of phagocytosis of the antigen with a mean vascular half life of 4 minutes as opposed to 2 minutes Therefore the action of

The notable exceptions are the skeletal system which must grow and remodel itself by increase in cell number and the derivatives of the RES the myeloid and lymphoid tissues

According to Heller (1958) there is not universal agreement that these tissues associated with the RES are in fact part of it but he believes that to consider them as such is both reasonable and customary If we accept this viewpoint there is a close anatomical and functional relationship between the system that collects the worn-out debris from the internal tissues (in a physiological state this is provided chiefly by the expendable cells corpuscles and particles of the peripheral blood and lymph) and the system that produces these same cells corpuscles and particles This relationship might be considered as an economic situation comparable to that of an island where the inhabitants eke out a precarious existence by taking in each other's washing Alternatively it could be classed as an admirable example of a good circumscribed biological cycle as now commonly recognized in biological chemistry although not yet fashionable in the philosophy of physiologists Nevertheless I do not quite understand why in the physiological state the myeloid and lymphoid tissues must be as active in the production of cells as in fact they can be shown to be

Red Cell Cycle

In man the average life span of the red corpuscles is about 100 to 120 days and virtually all of these bodies are in active circulation From the average life span the blood volume and the concentration of red cells in the blood production can be calculated to be 10^{10} cells per hour (Patt 1957) In the balanced state the loss of corpuscles must be the same and this seems to be due to some process of senescence that reaches a critical level after they have circulated for this period of about 100 days The corpuscles are not snatched at random from the circulating population by for instance marauding macrophages for the loss of marked erythrocytes from the circulation is linearly not exponentially related to time as would be the case with random loss (Callender *et al* 1945) Presumably this aging is due to chemical change It can be inhibited and brought almost to a stop *in vitro* by storage of blood at temperatures that nullify biochemical change for example −79 C the temperature of solid CO_2-ethanol mixture (Chaplin *et al* 1956) *In vitro* at the conventional temperatures of storage (4 C) the shelf life can be prolonged by providing additional substrates for various enzymes such as glucose (Rous and Turner 1916) or purine nucleosides (Prankerd 1956) and by adjusting the *p*H (Loutit *et al* 1943) However *in vivo* the cells are aged and removed after 100 days It is worth noting that a biochemical cycle then operates The iron containing moiety of the hemoglobin is preferentially reutilized in further hemoglobin synthesis (Finch *et al* 1949) although the globin fraction is thoroughly broken down (Jope 1946) Perhaps this process of continuous turnover of red corpuscles is a safety mechanism that facilitates rapid recovery from blood loss whether physiological as in menstruation or pathological as following injury

BIOCYCLES IN THE RETICULOENDOTHELIAL SYSTEM*

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The reticuloendothelial system (RES) is difficult to define with any degree of accuracy, and I shall not attempt to do so There appears, however, to be a general consensus that the network of reticulum cells in certain tissues such as spleen bone marrow, and lymphoid tissues, together with the littoral cells of the sinuses in those same tissues and in the liver, constitute a large part of it These cells are derived from the primitive mesenchyme, with the possible exception of those in that peculiar lymphoid mass the thymus the epithelial elements of which represent an outgrowth of primitive endoderm also some of its reticular cells are credited with a similar origin In addition descendent cells of the primitive mesenchyme, particularly in the neighborhood of blood vessels can be included in the RES This results *in the anatomical representation of the RES virtually all over the body*

Functionally the system is best identified by its capacity for scavenging debris hence its alternative name of the macrophage system (Maximov and Bloom 1952) It is thus intimately concerned with the physiological mechanisms of the body for the removal of effete cells

It is an interesting thought that the free living individual relying on his own resources has a certain amount of tissue as capital equipment built up in embryonic life from nutrients supplied by a maternal host and an additional quota of cells that are expendable and renewable

Almost all of these consumable stores are epithelia that is lining surfaces that are shed externally The skin is an obvious example The detritus of the mucosae of the respiratory tract is passed up from the bronchi or down from the nasopharynx by ciliary action to be swallowed It then joins the cells shed from the alimentary tract Some of this debris from the alimentary tract (which must be considerable if the time for complete turnover is about 2 days according to Leblond and Walker 1956) is presumably available for digestion with reabsorption of the breakdown products some is merely discharged in the fecal waste Epithelia shed from the genitourinary tract are similarly lost to the outside world Even the products of the germinal epithelium finally leave the body proper Thus all the valuable material is not available for re use or if it is only after digestion to its simplest con*stituents*

The cells as capital equipment be they of ectodermal origin such as the nervous system of endodermal origin such as liver parenchyma or of mesoblastic origin are tucked away in internal sites Then there is either no further turnover of cells as alleged for nervous tissue or replication only following abnormal loss of cells as in the hepatic parenchyma following necrosis (in fact repair of most lesions is effected by fibroblastic proliferation)

* Bicycle that commonly used term for an a[illegible]atic means of locomotion on two wheels is a misbegotten bastard of Latin (*bi*) and Greek [illegible] Further more it cannot be used for a cycle pertaining to life (*βίος*)

The fate of the granulocyte has not received recently at any rate any thing like the attention given to the study of the red cell Ambrus and Ambrus (1959) have followed some labeled granulocytes through the pulmonary al veolar wall to the bronchial secretion Do such leukocytes contribute to the nutrition of epithelia? In agranulocytosis it is epithelia that suffer In the past one has been impressed with the apparent lack of defence in epi thelia against bacterial invaders but are all of the facts known?

Whatever the validity of these suppositions may be it is unlikely that the great mass of leukocytes is lost to the body's economy If one takes the aforementioned figure of about 10^{10} cells produced per hour and assumes that each cell is a sphere of 10 μ diameter the total volume produced and lost per day would be about 100 cc Whether these cells die predominantly in travascularly or extravascularly most of their substance must find its way back to the RES to complete the cycle If a circle has a beginning the start ing point in marrow may be those cells that Cronkite *et al* (1959) call the primitive proliferating pool These cells include those called hemohistio blasts hemocytoblasts stroma cells stem cells primitive mesenchymal cells reticulum cells and histiocytes which in spite of the multiplicity of names represent but 1 or 2 per cent of the total cells of bone marrow Further more there appears to have been a similar early and considerable uptake into certain cells in connective tissue throughout the body presumably ac tive cells of the RES A normal turnover of 100 cc/day impressed me a quite a feat for a daily performance that still allows for bursts of speed over and above the ordinary routine

Lymphoid Cell Cycle

The lymphoid system of the mammal is notoriously still a mystery in spite of the volumes that have been written about it It is accepted by the International Commission on Radiological Protection (1955) to have a mass of 700 gm in the standard man weighing 70 kg that is about one half the mass of the red bone marrow which itself is one half the total bone marrow The lymph nodes and the collections of lymphatic tissue in the gut and else where receive by afferent lymphatics fluid from the peripheral tissue and deliver by efferent lymphatics which ultimately unite to form major trunks (for example the thoracic duct) discharging into the great veins It is gen erally accepted that the lymphocytes are produced in the so-called lymph follicles of the lymphatic tissue which has a reticuloendothelial network as background Here the most primitive recognizable lymphocyte—the large lymphocyte—is considered to give rise to medium sized lymphocytes which in turn produce small lymphocytes A cellular output of the tissue accom panies the effluent fluid and consists chiefly of small lymphocytes with fewer medium and still fewer large lymphocytes The rate of input of lympho cytes into the circulating blood can be estimated by sampling the largest duct the thoracic duct However not only is there the error of omission to measure the input of other lymphatic trunks but there also may be a direct entry of lymphocytes into the blood through the blood flowing through lymph tissue Yoffey *et al* (1959) believe this to be considerable The earlier evidence depended on differences in numbers all calculated on rather uncer

Granulocyte Cycle

The granular leukocytes are the next largest product of the myeloid tissue and are mainly the neutrophil leukocytes. Whereas I am satisfied that my physiologist colleagues understand the principal function of the red blood corpuscle which is to act as a common carrier of oxygen and CO_2 in the cycle between lung and tissue, I suspect that they have not yet identified the chief *raison d'être* of the neutrophil granulocyte. To pathologists such as myself the granular leukocytes are preponderantly living mobile defenders against bacterial invaders, but such a function need be called upon only when invasion in fact occurs. The granular leukocytes circulate at birth and in germ free animals. Perhaps natural selection has favored the provision of a natural defense mechanism of the body, a regular army always ready and not needing time for mobilization as do the reserves which defend by acquired specific immunity, but this seems an inadequate reason and the granular leukocytes could have routine functions. The pathologist again is impressed with the stickiness and the liquefactive properties of leukocytes even in aseptic inflammations and the collaborative activities of granulocytes and wandering macrophages of the RES are classically recognized. Granulocytes are a rich source of many enzymes other than the digestive, and I put forward the rather amorphous suggestion that the leukocyte is a common carrier of something. The reasoning is derived as follows.

The life span and distribution of these cells are known with much less certainty than are those of the red corpuscles. The life span in the peripheral blood has been estimated according to various experimental data as anything from a few hours to several weeks. Hamilton (1958) has noted, following Patt (1957) that the production of granulocytes by the marrow should by calculation be roughly the same as for red cells (nearly 10^{10} cells per hour). However if one assumes as Hamilton did a mean life of the cells in the peripheral blood of from 5 to 6 days one arrives at the conclusion that there must be about 50 times more granulocytes in the body than are circulating in the blood but histologists do not find evidence of such substantial concentration. On the other hand if one accepts the hypothesis that the mean life in the blood is not 5 days but rather in the region of 5 hours which is the rate of loss of granulocytes from the blood after near lethal whole body irradiation when the marrow reserves are exhausted after a few days, the conclusion is that there is little reserve outside the marrow (Patt 1959), although there may be leukocytes sequestered along the walls of blood vessels. Furthermore according to Patt's (1959) data on H^3 labeled cells not only is the life span in the peripheral blood short but the cells are lost in an exponential fashion. What can be the reason for an enormous production and a random loss? These cells unlike you and me and the red corpuscles are not doing a daily job until worn out when they are replaced by other workers. It seems that they function by their death rather than by their life: they must be providing a service to some tissue that cannot be done with its own materials. This tissue might be the vascular endothelium with which many leukocytes are in contact or since the leukocyte is ameboid it could be an extravascular tissue. One could speculate with more confidence if one had more data on the fate of these leukocytes.

be reutilized without undue degradation by successive generations of the e cell On this bi is Medawar (1957) has built a further hypothesis of the pos ible function of the lymphocyte as a recycling carrier of DNA from pe ripheral ti ue to lymphatic ti sue and back again but little further evidence seems to have come to light in favor of either concept

Whatever the whole truth about the postulated and long peripatetic life of the lymphocyte may be its fate i probably once again quietly to die inter nally It is true that some lymphocytes usually can be seen histologically in the intestinal mucosa where according to Trowell (1952) they may have acquired some different properties such as increased resi tance to X irradia tion and where Ambrus and Ambrus (1959) confirm that they may be lo t to the lumen but pyknotic and pre umably dying lymphocytes frequently can be seen in ti ues (Trowell 19 8a) and e pecially in lymph nodes This is circum tantial evidence of recycling within the lymphatic system

If we may return to armchair speculation and calculation Hamilton (1958) a ume that one half the 100 gm of lymphoid ti sue is compo ed of small lymphocytes and derives a figure for the whole body of 14×10^{11} such cells as compared with 11×10^{9} in the circulation and a daily output of the tho racic duct of $16\ 8 \times 10^{9}$ He further assumes an average life cycle of 21 days and concludes that the daily production is $6\ 5 \times 10^{10}$ cells a day In a balanced state thi means that a imilar number is destroyed If the small lymphocyte i a sphere with a diameter of 8 μ the cell destroyed amount to about 15 cc /day This normal destruction i to be contrasted with the abnormal rate following exhibition of cytotoxic agents physical (X rays for example) or chemical (the nitrogen mustards) when virtually all lympho cyte die within a matter of hours This de truction must involve hundreds of grams of lymphocytes and the RES must deal also with simultaneous destruction of bone marrow In pite of the insult the RES digests with avidity In such cases the con umption of what may amount to 2 kg of tissue does not re ult in significant lo s of nitrogen from the body which speaks well for the con ervative powers of the RES in the total economy

Interaction Between Cycles

The bone marrow and the lymphatic tissue have the common property of di charging their cellular end products to the peripheral blood These struc tures are related embryologically in their origins from the primitive me en chyme It was once a fashionable exercise to deduce from morphologic evi dence whether in the mature animal all of the products came from a single type of stem cell (the monophyletic hypothe is) or whether each line of cells—erythrocyte granulocyte monocyte and lymphocyte—had separate precur sive bla t cells (the polyphyletic hypothe is) Certainly the lines are ac cepted a di tingui hable at an early definitive stage for example the pro normoblast and the promyelocyte the controversy concerned the earlier blast cell stage It now eem at any rate in pathological tates such as leukemia that myeloblastic lymphobla tic and monobla tic types exist These types have differences detectable cytochemically (Hayhoe 1959) functionally (Pul vertaft and Humble 1959) and by therapeutic re pon e (*see* Bethell 1957)

tain and unconfirmable bases The supporting data came from measurements of desoxyribonucleic acid (DNA) turnover with P^{32} Suffice it to say that this additional direct entry has not been measured with precision

Furthermore, we have that other mysterious tissue the thymus, which is lymphoid in structure but has different origins and certainly some different functions from the rest of the lymphoid system It is an active tissue in the young and atrophic in the mature Its epithelial remnants may be responsible for a lymphocytosis stimulating factor' (Metcalf 1959) and for the important role of the thymus in the development of lymphatic leukemia, particularly in the mouse However, its lymphoid tissue produces morphologically typical small lymphocytes in the cortex whence, according to Sainte Marie s (1959) interpretation of the histology, they migrate to the medulla and then enter the blood vessels by diapedesis

The measurable daily input of lymphocytes into the circulation gives a daily replacement factor of between 1 for man and 11 for the rat these must necessarily be minimum values The problem is when the lymphocytes leave the circulation do they do so permanently or do they return and recirculate, perhaps after some form of reconditioning? Gowans (1959) in particular, argues the case for recirculation The evidence is based largely on his own experience following Mann and Higgins (1950) that blood lymphocytes progressively decline in number following continuous drainage of fluid from the thoracic duct but that reinfusion of the fluid restores the level Recycling a cellfree fluid or fluid in which the cells have been killed by ultraviolet light does not prevent the fall On the other hand infusion of lymphocytes into the blood increases the concentration of these cells in the outflow of the thoracic duct and if the infused cells are labeled with P radioactive lymphocytes soon appear in thoracic duct lymph

This hypothesis of recirculation indicates merely that the lymphocytes have in fact a substantially long life while arithmetical considerations alone (for example, calculations of daily replacement factors) suggest they have a life of only a few hours The now much quoted work of Ottesen (1954) and Hamilton (1956) on labeling the cells *in vivo* with P^{32} and C^{14} respectively similarly indicated a long life in some instances a very long life for lymphocytes What the cells do when they are not circulating in the blood stream is unknown but sparsely scattered lymphocytes are a common finding in the normal histological picture of many tissues At present one must presume merely that with their small size and active movement they are recycling between blood and tissue thereby carrying on some unknown biochemical reversible process My interpretation of figures provided by Hamilton (1959) and by Schooley *et al* (1959) is that destruction or loss of lymphocytes is also a random process which again may indicate that their function is associated with their death In some circumstances they may be associated with division of tumor or tissue cells as seen cinematographically by Humble *et al* (1956)

Hamilton s (1956) alternative hypothesis to account for his experimental findings suggested that the lymphocytic cell might not live as long as his data indicated instead the DNA into which his C^{14} adenine was built might

they may become granulocytes erythroblasts macrophage and so on They may be looked upon as an easily movable mesenchymal reserve Yoffey *et al* (1958) have taken the concept further While not adhering to the idea of recirculation of lymphocytes as such Yoffey has been impressed both by the large numbers and the distribution of lymphocytes in the intersinusoidal parenchyma of the bone marrow It is generally accepted that in mammals there is no organization for the production of lymphocytes in marrow as there is in lymph tissue Consequently the cells as Yoffey claims probably enter the marrow by diapedesis from the sinuses Yoffey interprets the histological and experimental findings of his group as indicating that the small lymphocyte passes through transitional stages of dedifferentiation to a blast cell of the marrow This proposed cycle provides good reasons for the presence of such a high concentration of lymphocytes in the marrow as compared with other tissues

However unless the ultimate blast cell produced is a temporarily resident lymphoblast rather than a multipotent hemocytoblast one has difficulty in explaining the aforementioned transfusion experiments in lethally irradiated animals In these there was a one way traffic of developing cells from marrow to lymph tissue but none in the reverse direction It is more tempting to reflect with Medawar (1957) that although the lymphocytes in marrow have not been seen to expire there (nor can the nuclei of normoblasts) they are there to give up if not their intact DNA and nuclear antigen to newly forming marrow cells at least some of the essential constituents The recent observation of Schooley *et al* (1959) that H³ marked small lymphocytes selectively accumulate in bone marrow may indicate that this is their last resting place either before dissolution or before transformation in the sense that Yoffey *et al* (1958) imply

While recent dogma certainly leads one to regard the small lymphocyte as a cell at the end of a chain rather than part of a cycle there is the direct observation of my colleague Trowell (1958*b*) that lymph nodes in culture *in vitro* may show phagocytic reticulum cells containing debris of lymphocytes and developing into large lymphocytes Trowell therefore propounds lymphocyte macrophage cycles one involving this reutilization the other (also suggested from his culture) of lymphocyte transformation through monocytes to reticulum cell

Cycles in the Mechanism of Immunity

While we cannot be certain with what the lymphatic system normally occupies itself there is a long accepted basis for its being concerned with the defence mechanism against foreign invaders First the lymph collections can act as mechanical filters but of equal or greater importance is their role in specific immunity to antigens Such specific immunity may involve the production of humoral substances (antibodies) detectable by one or other of the now classic means for their identification such as precipitation agglutination and complement fixation Alternatively it may be manifest only by the production of sensitized cells

There now seems to be widespread agreement with Fagraeus (1948) that the

Nevertheless, common stem cells may still exist not necessarily being active normally but capable of being called into operation at need For instance following destruction of both myeloid and lymphatic tissues of mice by the LD_{100} of X rays a recolonization of the depleted tissues can be effected by injecting intravascularly suspensions of hemopoietic tissue such as the bone marrow of the adult or the liver of the fetus No lymphopoiesis is recognized as taking place in these tissues nevertheless the suspensions recolonize both myeloid and lymphoid tissues (Ford *et al* 1956) Adult marrow and fetal liver must therefore contain stem cells for both the myeloid and lymphoid series or they contain in addition to myeloid precursors lymphoid cells that can dedifferentiate to large lymphocytes Suspensions of lymph nodes have not been effective in our experience or in scanty reports in the literature in preventing the death of lethally irradiated mice There is indirect evidence that they recolonize lymph tissue Thus host type lymphocytes given with homologous bone marrow cells preclude the survival of such animals as would be expected from the administration of the bone marrow (van Bekkum and Vos 1957) Presumably the lymphoid cells adoptively restore the hosts lost immunological reactivity against the homologous marrow cells My colleagues (Ford *et al* unpublished data) have direct evidence of an initial recolonization of this type For example irradiated CBA mice given isologous bone marrow from other CBA animals and lymphocytes from the F_1 cross CBA × T6 (T6 being a line containing a chromosomal translocation that acts as a visible marker in mitotic figures) have the T6 marker in mitotic cells of lymph nodes in the early stages of recovery

Further evidence of monophyletic potential comes from other cytological studies Mice irradiated with rather less than the LD_{100} (about LD_{80}) when resuscitated with foreign bone marrow are initially recolonized by it but after a few months may undergo reversion of their myeloid and lymphoid tissues to host type cells These cells in mitosis not infrequently show radiation induced chromosome translocations each individual translocation being specifically identifiable Thus clones of cells are recognizable as stemming from a single mother cell with its characteristic lesion The members of a clone are found in both myeloid and lymphoid tissues which indicates that under these nonphysiological conditions monophyletic development can occur (Barnes *et al* 1959)

These findings indicate that under appropriate conditions there is a metastasis of active cells presumably by the blood stream In the past metastasis was considered a phenomenon confined chiefly to malignant cells Bond and his group (1958) with their technique involving thymidine uptake have identified that some 0 05 per cent of circulating leukocytes will take up the labeled thymidine *in vitro* These cells usually have the morphologic appearance of monocytes It is tempting to postulate that if this uptake really identifies DNA synthesis in the nucleus rather than clearance by the cytoplasm these cells are the putative cycling agent

The free movement and circulation of such multipotent cell however was propounded by Maximov followed by Maximov and Bloom (1952) who considered such cells to be lymphocytes Under normal condition they keep the appearance of lymphocytes but in response to certain pathological stimuli

tantamount to somatic mutation of the genetic material. If 10^4 variants are required (certainly a possible number according to Burnet, 1959a) and if the maximum mutation rate is 10^{-4}, 10^8 cell generations would be the minimum required. It is possible that the number of generations of primitive mesenchymal cells is accomplished by birth in the mouse (approximate weight 1 gm.) and by a relatively early stage of embryonic development in man. However, what is important in the present context are the mechanisms in the free living animal exposed to foreign antigen. Burnet, having noted the evidence of longevity and recirculation of lymphocytes, considers that the most likely process is that foreign antigen makes contact with lymphocytes of the appropriate clone with the ultimate result that the clone is specifically stimulated. All of the possible cycles through which lymphocytes may operate are thus, according to this theory, potential means for ensuring an immune response and for perpetuating immunological memory.

This hypothesis certainly accomplishes the object of explaining immunological tolerance of self (and autoimmune disease—Burnet, 1959b) and memory, but it places an extreme weight on somatic mutation that may be difficult of acceptance to many. It leaves much to speculation, but like all good hypotheses it opens many avenues for investigational exploration.

Summary

One cannot summarize a circumlocutory review such as this; one can, however, identify six points and the questions that they suggest.

(1) The consumable stores of body cells are mainly epithelia shed to the outer world. The exception is blood; this is produced by the RES, which also collects its debris.

(2) Red corpuscles have a fixed life span in the peripheral blood. What determines this life span?

(3) Neutrophil granulocytes are produced in about the same quantity (as mass per day) as red corpuscles. They leave the circulating blood in random fashion within a few hours. Why?

(4) Lymphocytes are concerned with the defense mechanisms of the body. How? They recirculate and finally leave the circulation randomly. Are they transformed or are their products reutilized?

(5) Under certain conditions stem cells can be shown to produce both myeloid and lymphoid progeny (monophylogenesis). Is this the normal physiological condition or does one system beget the other?

(6) Lymphoid tissue and primitive mesenchyme are concerned with immunological defense. What are the steps involved in both humoral and cellular responses?

References

Ambrus, C. M. & J. L. Ambrus. 19 9. Regulation of the leukocyte level. Ann. N. Y. Acad. Sci. 77(3): 445.

Barnes, D. W. H., C. E. Ford, S. M. Gray & J. F. Loutit. 19 9. Spontaneous and induced changes in cell populations in heavily irradiated mice. In Progress in Nuclear Energy, Biological Sciences, Ser. VI, 2: [illegible] Pergamon Press. London, England.

Berrian, J. H. & L. Brent. 1958. Cell bound antibodies in transplantation immunity. Ann. N. Y. Acad. Sci. 73(3): 654.

elaboration of the humoral antibody depends on the chain of development from RE cell through plasma blast to plasma cell and that antibody production is maximal when the chain is at the transitional stage before the final stage of plasma cell (of whose fate I am ignorant). Production of plasma cells is not confined to lymph tissue.

On the other hand, the sensitized cells that appear to be responsible for delayed supersensitivity (for example, reaction to tuberculin) and for transplantation immunity are not identified with certainty, but there are grounds for belief that their origins are confined to lymphatic tissue and that they are the common lymphocytes. Perhaps these sensitized cells contain some endoantibody that can be liberated only on lysis of the cell, as distinguished from the humoral exoantibody of the transitional plasma cell. Berrian and Brent (1958) have described some cell-bound antibodies in transplantation immunity.

The machinery of immunity still requires elucidation and is a subject for speculation. Burnet (1959*a*) notes that "there are two sets of observable phenomena which are crucial to the understanding of antibody production. (i) The non-antigenicity of the body components and the phenomena of prenatally induced tolerance. (ii) The persistence of immunological memory over many years."

Hamilton (1958) has offered an explanation of this long "memory" from his own, Trowell's, and similar data. Cycling by reutilization of the DNA of the lymphocytes could provide, he suggests, a template that survives beyond the life of the individual cell in which antigen first stimulated production of antibody. This is a variant of the Haurowitz-Pauling direct template theory, but it seems to me to imply that antigen originally modified the DNA of the reactant cell, causing what is virtually a directed mutation. Burnet and Fenner (1949) also once propounded an indirect template theory in an attempt to explain immunological self-tolerance, since this was not covered by the direct template theory. It was necessary to postulate "self markers" in fetal cells that were broken down when effete by the immature RES, in which hypothetical "recognition units" were thereby induced. The union of self markers and recognition units developed and fixed biochemical cytoplasmic processes for the most profitable disposal of the residues, and these processes became like plasmagenes transmissible to daughter cells. In the later free-living state the animal's RES would thus be self-tolerant, but foreign antigens would excite other processes, thus leading to production of antibodies and immunity.

Burnet (1959*a*) has now accepted the fact that this indirect template theory had some clumsy features. Instead, he has put forward a "clonal selection" theory, which demands that for each antigenic configuration there is originally a primitive cell of the RES that can develop by division into a clone of cells that produce the corresponding antibody. Antigenic configurations already represented by heredity in the developing fetus result in the suppression (and probably the death) of the corresponding clone mother cells. This theory explains immunological tolerance of self as adequately as the former indirect template theory and, in addition, the experimental observations of one cell—one antibody (Nossal and Lederberg, 1958), but it implies that at some early stage of fetal development there is an extreme diversification of cells of the RES

PULVERTAFT, R. J. V. & J. C. HUMBLE (19[illegible]). The culture of bone marrow in acute leukaemia. 7th European Congr. Haematol. Abstr. 106.

R[illegible], [illegible] & J. [illegible] TURNER (1916). Preservation of living red blood corpuscles in vitro. J. Exptl. Med. 23, 219.

SAINTE-MARIE, G. (19[illegible]). Lymphocyte formation in the thymus of the rat. Proc. 3rd Canadian Cancer Conf. 337. R. W. Begg, Ed. Academic Press. New York, N.Y.

SCHOOLEY, J. C., B. J. BRYANT & L. [illegible] KELLY (19[illegible]). Preliminary autoradiographic observations of cellular proliferation in lymphoid tissues using tritiated thymidine. In The Kinetics of Cellular Proliferation. 208. F. Stohlman, Ed. Grune & Stratton. New York, N.Y.

TROWELL, O. A. (1952). The sensitivity of lymphocytes to ionizing radiation. J. Pathol. Bacteriol. 64, 687.

TROWELL, O. A. (19[illegible]a). The lymphocyte. Intern. Rev. Cytol. 7, 235.

TROWELL, O. A. (1958b). Some properties of lymphocytes *in vivo* and *in vitro*. Ann. N.Y. Acad. Sci. 73(1), 105.

VAN BEKKUM, D. W. & O. VOS (19[illegible]7). Immunological aspects of homo- and heterologous bone marrow transplantation in irradiated animals. J. Cellular Comp. Physiol. 50 (Suppl. 1), 139.

YOFFEY, J. M., G. A. HANKS & L. KELLY (19[illegible]8). Some problems of lymphocyte production. Ann. N.Y. Acad. Sci. 73(1), 4.

BETHELL F H 1957 Chairman Round table discussion on therapy *In* The Leukaemias Etiology Pathophysiology and Treatment 619 J W Rebuck Ed Academic Press New York N Y
BOND V P T M FLIEDNER E P CRONKITE J R RUBINI G BRECHER & P K SCHORK 1958 Deoxyribonucleic acid synthesizing cells in the peripheral blood of normal human beings Science 128 202
BURNET F M 1959a *In* The Clonal Selection Theory of Acquired Immunity 67 Cambridge Univ Press Cambridge England
BURNET F M 1959b Auto immune disease Brit Med J ii 645
BURNET F M & F FENNER 1949 The Production of Antibodies Rev ed Macmillan Melbourne Australia
CALLENDER S T E I O POWELL & L J WITTS 1945 The life span of the red cell in man J Pathol Bacteriol 57 129
CHAPLIN H JR H CRAWFORD M CUTBUSH & P L MOLLISON 1956 Preservation of red cells at −79 C Clin Sci 15 27
CRONKITE E P T M FLIEDNER V P BOND J R RUBINI G BRECHER & H QUASTLER 1959 Dynamics of hemopoietic proliferation in man and mice studied by H³ thymidine incorporation into DNA Ann N Y Acad Sci 77(3) 803
FAGRAEUS A 1948 Antibody production in relation to the development of plasma cells Acta Med Scand 130 Suppl 204
FINCH C A J A WOLFF C E RATH & R C FLUHARTY 1949 Iron metabolism erythrocyte turnover J Lab Clin Med 34 1480
FORD C E J L HAMERTON D W H BARNES & J F LOUTIT 1956 Cytological identification of radiation chimaeras Nature 177 452
GOWANS J L 1959 Life history of lymphocytes Brit Med Bull 15 50
HAMILTON L D 1956 Nucleic acid turnover studies in human leukaemic cells and of the function of lymphocytes Nature 178 597
HAMILTON L D 1958 Control and functions of the lymphocyte Ann N Y Acad Sci 73(1) 39
HAMILTON L D 1959 Carbon labelling of DNA in studying hematopoietic cells *In* The Kinetics of Cellular Proliferation 151 F Stahlman Ed Grune & Stratton New York N Y
HAYHOE F G J 1959 Cytochemical techniques in diagnostic haemotological cytology 7th European Congr Haematol Abstr 103
HELLER J H 1958 Measurement of the function of the reticuloendothelium Ann N Y Acad Sci 73(1) 212
HUMBLE J C W H M JAYNE & R J V PULVERTAFT 1956 Biological interaction between lymphocytes and other cells Brit J Haematol 2 283
INTERNATIONAL COMMISSION ON RADIOLOGICAL PROTECTION 1955 Recommendations Brit J Radiol Suppl No 6
JOPE E M 1946 The disappearance of sulphaemoglobin from the circulating blood in relation to red cell destruction Proc Roy Soc Med 39 760
LEBLOND C P & B E WALKER 1956 Renewal of cell populations Physiol Revs 36 255
LOUTIT J F P L MOLLISON & I M YOUNG 1943 Citric acid sodium citrate glucose mixtures for blood storage Quart J Exptl Physiol 32 183
MANN J D & C M HIGGINS 1950 Lymphocytes in the circulation intestinal and hepatic lymph Blood 5 177 190
MAXIMOW A A & W BLOOM 1952 *In* A Text Book of Histology 6th ed 89 Saunders Philadelphia Pa
MEDAWAR P B 1957 Transplantation immunity and subcellular particles Ann N Y Acad Sci 68(2) 255
METCALF D 1959 The thymus its physiology stimulating factor and its relation to lymphatic leukaemia Proc 3rd Canadian Cancer Conf 1958 351 R W Begg Ed Academic Press New York N Y
NOSSAL G J V & J LEDERBERG 1958 Antibody production by single cells Nature 181 1419
OTTESEN J 1954 On the age of human white cells in peripheral blood Acta Physiol Scand 32 75
PATT H M 1957 A consideration of myeloid erythroid balance in man Blood 12 777
PATT H M & M A MALONEY 1959 Kinetics of neutrophil balance *In* The Kinetics of Cellular Proliferation 201 F Stahlman Ed Grune & Stratton New York N Y
PRANKERD T A J 1956 The revival of stored blood through guanosine and its successful transfusion Lancet 1 469

Ellvertapt K J V & J C Humble 1959 The culture of bone marrow in acute leukaemia 7th European Congr Haematol Abstr 106

Rous P & J R Turner 1916 Preservation of living red blood corpuscles *in vitro* J Exptl Med **23** 219

Sainte Marie G 1959 Lymphocyte formation in the thymus of the rat Proc 3rd Canadian Cancer Conf 337 R W Begg Ed Academic Press New York N Y

Schooley J C B J Bryant & L S Kelly 1959 Preliminary autoradiographic observations of cellular proliferation in lymphoid tissues using tritiated thymidine *In* The Kinetics of Cellular Proliferation 208 F Stahlman Jr Ed Grune & Stratton New York N Y

Trowell O A 1952 The sensitivity of lymphocytes to ionizing radiation J Pathol Bacteriol **64** 687

Trowell O A 1958*a* The lymphocyte Intern Rev Cytol **7** 235

Trowell O A 1958*b* Some properties of lymphocytes *in vivo* and *in vitro* Ann N Y Acad Sci **73**(1) 105

van Bekkum D W & O Vos 1957 Immunological aspects of homo- and heterologous bone marrow transplantation in irradiated animals J Cellular Comp Physiol **60** (Suppl 1) 139

Yoffey J M C A Hanks & L Kelly 1958 Some problems of lymphocyte production Ann N Y Acad Sci **73**(1) 47

Part III The Relations of the Reticuloendothelial System to Infection and Immune Reactions

THE RETICULOENDOTHELIAL SYSTEM IN ANTIBODY FORMATION

Robert W. Wissler, Frank W. Fitch

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Mariano F. La Via*

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Introduction

The important role played by mesenchymal cells in combating infection and forming antibody is now generally accepted. There can be little debate about the three following basic observations:

(1) Macrophages (fixed and free) avidly ingest and frequently kill and digest living microorganisms.

(2) Antibody forming cells arise from more or less primitive cells (reticular cells) and, under some conditions at least, they develop into plasma cells that contain antibody.

(3) Small or medium sized lymphocytes have an important function in the immune process and in delayed hypersensitivity.[1]

Beyond these generally accepted concepts many questions remain. For example, if one accepts the coexistence of 2 kinds of primordial reticuloendothelial cell, one of which is phagocytic (fixed macrophage) and the other of which is not (primitive reticular cell), what is the interrelationship of these cells and how does each participate in resisting infection and forming antibody? How is a mesenchymal cell stimulated to form antibody? Are mitoses commonly or only rarely necessary if antibody formation is to take place? What is the usual fate of the antibody forming cell?

Some idea of the state of flux that has characterized the relation of cells to antibody formation may be gained by a brief consideration of the rapid shifts of scientific opinion during the past quarter century (TABLE 1). Evidence has been advanced to support the formation of antibody by macrophages (histiocytes), by lymphocytes, and by plasma cells or their precursors, but most of this evidence has been indirect. There has been a lack of agreement on the nomenclature and the possible interrelationships among the various mesenchymal cells. This difficulty remains, but it is probably less of a deterrent to progress than it was two decades ago.

Burnet's recent lucid lectures on *The Clonal Selection Theory of Acquired Immunity*[2] outline in broad bold strokes the problem faced in relating cell to antibody formation. Burnet points out that any theory of how cells fabricate antibody must take into account the important chronological and quantita-

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tive differences between the primary and secondary immune responses as well as other phenomena of the anamnestic response Furthermore the theory should explain the presence of local tissue immunity adjuvant action and immunological tolerance The e lectures offer a splendid foundation for any consideration of the role of cells in immune reactions

The principal purpose of this paper is to reevaluate the problem of how cells *form antibody* Recent observations are *correlated* with the clonal selection theory No attempt is made to provide final answers or to review the subject comprehensively since excellent recent reviews already exist [8] An earnest effort is made to correlate some of numerous observations that have been reported from various laboratories and to clarify interrelationships among the cells involved but most significantly an attempt is made to project these

TABLE 1

SUMMARY OF EVIDENCE IMPLICATING VARIOUS CELLS IN ANTIBODY FORMATION

(1) Macrophages
- (a) Cells that ingest antigen might be expected logically to form antibody
- (b) Blockade of RE system *decreases antibody response*
- (c) Cytoplasmic shedding of macrophages correlates with antibody formation
- (d) Tissue depots with large proportions of macrophages show high concentration of antibody

(2) Lymphocytes
- (a) Lymphocytes increase in tissue during infection
- (b) Antibody titer increases acutely when lymphocytes break up (ACTH cortisone)
- (c) Antibody can be extracted from lymphosarcoma
- (d) Antibody concentration is higher in lymph node than in serum
- (e) Antibody forming capacity can be transferred by lymph node cells

(3) Plasma cells
- (a) Plasma cells increase in chronic infection
- (b) Hyperglobulinemia correlates with number of plasma cells in tissue
- (c) Hyperimmunization of rabbits increases number of plasma cells in tissue
- (d) Proliferation of immature plasma cells in spleen and lymph node of rabbit occurs during antibody formation
- (e) No plasma cells are present in agammaglobulinemia
- (f) Antibodies have been demonstrated inside plasma cells using fluorescein labeled antigen

observations into a meaningful picture that possibly could improve under*standing and help to guide future investigation*

Interrelationships of Cells

It is impossible to reason in this field without some concept of the interrelationships existing among the mesenchymal cells involved in antibody formation Some cell transformations are agreed upon while others are still in *doubt* There appears to be considerable general agreement The schematic arrangement presented in FIGURE 1 has many points in common with that recently proposed by Marshall but it must be emphasized that in many areas it is presumptive rather than definitive Furthermore no special *brief is held for* the terminology employed As in the scheme published by *Marshall* the diagram presents the fibroblast osteoblast *erythrocyte granulocyte* [illegible] and plasma cell as end stage cells incapable *of reversion* [illegible]

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"secondary" reaction or with other types of antigens is from small or medium lymphocyte to macrophage to plasma cell (A to B to C)

Factors Influencing the Cellular Patterns in Antibody Formation

A number of factors have an important influence on the type of cellular reaction that follows antigenic stimulation Some of these such as total body irradiation[8, 9] chronic protein deprivation[10] acute interference with protein synthesis[11] and cortisone administration[1, 13] have been studied Other factors studied less completely may alter greatly the pattern of cellular response after administration of antigen These include the physical state and chemical nature of the antigen whether the spleen (intravenous injection) or lymph node (subcutaneous injection) is the site of antibody formation being studied whether the cellular reaction is studied after the first injection or after several injections of a given antigenic substance (or substances) and the species being utilized In general there have been very few studies in which these parameters have been evaluated adequately under otherwise constant conditions In the paragraphs to follow we attempt to compare some of the results that we have obtained using one set of experimental conditions with the results obtained by others who have often used quite different experimental designs At present some of the variations in cellular reactions observed under differing conditions appear to defy analysis but in other instances as outlined later in this paper it seems possible to construct a rational explanation of the differences in cell response described

Observations in the Rat Spleen Following a Single Intravenous Injection of a Particulate Antigen

The cellular reaction of the rat spleen to a single antigenic stimulation has been studied extensively in this laboratory[8, 11, 14] The rat spleen was chosen for five reasons namely (1) with the dose of particulate antigen used it appeared to produce most if not all of the humoral antibody following a single intravenous injection[15, 16] (2) the antigen localized in the spleen very quickly thus providing a brief accurately timed stimulus[8, 10] (3) the initial tissue localization of the particulate antigen was definite and could be traced easily[8, 10] (4) the cellular reaction could be interpreted with little difficulty by means of closely spaced histological observations of the spleen[14] and (5) the weight and chemical composition of the organ were easy to obtain[17]

During the past 6 years only one important variation in the cell pattern of response has been noted in this laboratory in studies of the spleen following injection of one or another of several different particulate antigens This consists of the presence or absence of very acute reactions in the splenic follicle[17] and is illustrated in FIGURE 2 Here it is evident that when a suspension of an endotoxin containing organism such as killed *Salmonella typhosa* is injected intravenously there is considerable reaction of the malpighian corpuscle within the first 24 hours This reaction is absent when a bland antigen such as washed sheep erythrocytes is used In both instances however peak humoral antibody titer (agglutinins to H antigen and hemolysin to sheep cells) the timing and shape of the antibody curve and the other cellular reactions are

another cell type. This scheme differs from Marshall's diagram in that the lymphocytes are regarded as multipotential cells capable of developing into macrophages and/or plasma cells and possibly into hemocytoblast. This difference in concept is important since observations in this laboratory suggest that the small or medium sized lymphocyte is the "messenger" cell that carries

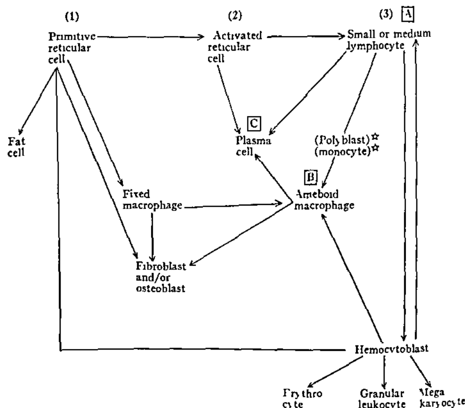


FIGURE 1. Interrelationships among mesenchymal cells. Solid arrows indicate transformations for which there is general agreement. Broken arrows, suggested transformations. The numbers designate the cell progression from 1 to 3 that has been observed in the rat spleen following a single intravenous injection of a particulate antigen. The letters are the suggested cell transformations from A to C occurring following a second (or repeated) injection of antigen.

information throughout the body regarding previous experience with antigens. Later in this paper it will be evident that such a cell is a necessary link in the process and that the plasma cell is unlikely to play this role. In the rat spleen following a single intravenous injection of particulate antigen the principal steps of the cellular progression accompanying antibody formation that we have observed repeatedly are from reticular cell to activated reticular cell to small or medium sized lymphocyte (1 to 2 to 3, *see* FIGURE 1). In contrast, the pattern of cell development described by several other observers during the

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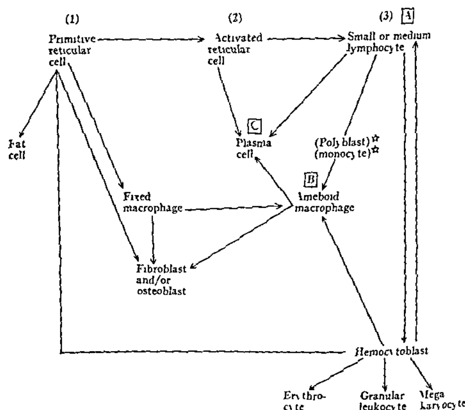
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☆ Transitional cell

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FIGURE 3 Proposed cellular patterns during primary (A) and secondary (C) immune response following intravenous particulate antigen injection The diagrams of the spleen indicate the relative participation of the splenic red and white pulp during (A and C) and between (B) these 2 types of responses The proposed cell transformations relative to mitotic activity the types of cells implicated and the timing of the various reactions relative to antibody release are indicated in the cell sequences shown under the spleens labeled A and C



FIGURE 1. Lymphoid follicle of the rat spleen. Methyl green pyronine stain. ×250. (A) From a normal rat showing "resting" follicle. (B) From a rat sacrificed 12 hours after typhoid vaccine intravenously showing the great follicular activity including some decrease in small lymphocytes in the central portions of the follicle and some increase in the relatively pyroninophilic lymphoblasts near the edge of the follicle. (C) From a rat sacrificed 12 hours after the intravenous injection of sheep erythrocytes showing little or no follicular activity. Spleen from animals from the same experimental group sacrificed 4 days after injection of the same antigen showed many pyroninophilic cells in the red pulp.

a ingle injection of antigenic material then the rat pleen response to different particulate antigen i quite con istent and is limited to the red pulp It is hown diagrammatically in FIGURE 3a

The evidence indicates that most if not all of the antibody is formed in the spleen and that the antigenic particles are engulfed by macrophages (histiocytes) that promptly digest them at least to the extent that an I[131] label is released At thi tage none of the antigen i deposited in the white pulp

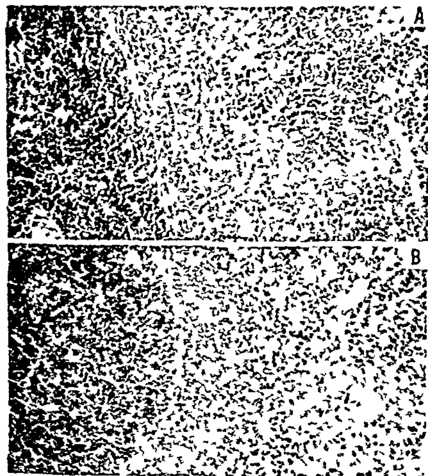


FIGURE 5 Rat spleen (edge of foll cle marginal zone and red pulp) at different intervals after the intra enous injection of a flagellar preparation of *Salmonella typhi* Most of the changes a e in the red pulp with littl s gnificant acti ation in the white pulp (follicles) Methyl green pyronine stain ×350 (A) Six d ys after injection The red pulp hows many small dark cells and a great reduction in the large pyroninophilic c lls as compared to the sit uat on 2 days earlier (FIGURE 4B) The follicle is n t altered (B) Ei ht days after injection. The red pulp has returned to the pre njection state (FIGURE 4A) except f r the presence of an occasional plasma cell.

quite similar Therefore we have concluded that the reaction of the follicle is not necessary for antibody response in the rat When it occurs it probably reflects an effect of liberated toxic substances (endotoxin?) rather than an

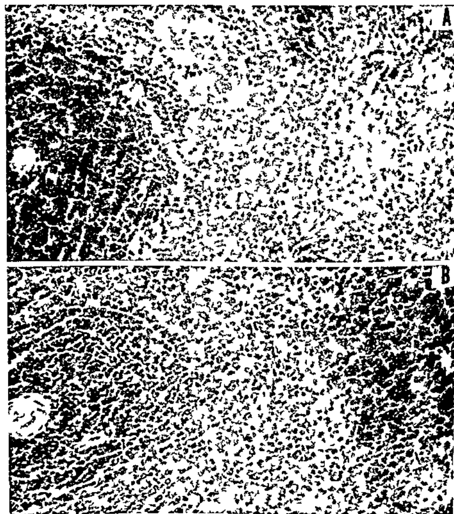


FIGURE 4 Rat spleen (edge of follicle marginal z ne and red pulp) at different intervals after the intravenous injection of a flagellar p epa ation of *Sal nonella typhi* Most of the changes are in the red pulp ith littl significant cti ation in the white pulp (follicles) Methyl green pyronine stain ×350 (A) N rmal at n te the relative emptiness of the red pulp (B) Four days after inj cti n N te the similarity of the follicle and the marginal zone to that of the control spleen (A) See FIGURE 5

integral part of the antibody re pon e Spleen weights correlate well with these ob ervations[14] and the presence of numerou neutrophilic leukocytes in the sinuses of the splenic red pulp during the first 12 to 24 hours after the injection of toxic antigens[17] supports this interpretation

If one excludes this single variable a pect of the cellular respon e fol

a single injection of antigenic material then the rat spleen response to different particulate antigens is quite consistent and is limited to the red pulp It is shown diagrammatically in FIGURE 3a

The evidence indicates that most if not all of the antibody is formed in the spleen and that the antigenic particles are engulfed by macrophages (histiocytes) that promptly digest them at least to the extent that an I[131] label is released At this stage none of the antigen is deposited in the white pulp

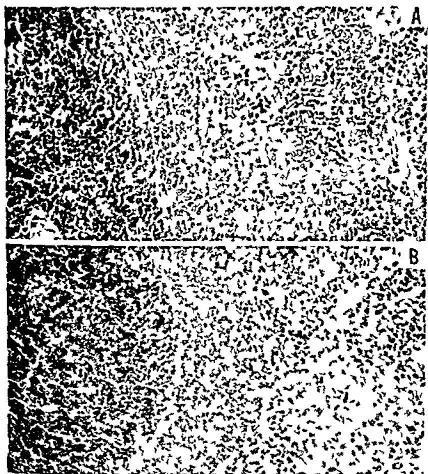


FIGURE 5 Rat spleen (edge of follicle marginal zone nd red pulp) at different intervals after the intravenous injection of a flagellar preparation of *Salmonella typhi* Most of the changes are in the red pulp with little significant activation in the white pulp (follicles) Methyl green pyronine stain ×350 (A) Six days after injection The red pulp shows many small dark cells and a great reduction in the large pyroninophilic cells as compared to the situation 2 days earlier (FIGURE 4B) The follicle is not altered (B) Eight days after injection The red pulp has returned to the preinjection state (FIGURE 4A) except for the presence of an occasional plasma cell

Radioautographic evidence indicates that all of it is deposited in the red pulp, about one half in the marginal zone at the edge of the follicles and the remainder in the rest of the red pulp.[8, 9]

The micrographs of FIGURES 4 and 5 are derived from experiments recently performed by H. Kattlove, M. F. La Via, and A. E. Warren (unpublished observations) using a typhoid flagellar preparation as the intravenous antigenic stimulus. The results are quite similar to those obtained previously with more complex antigenic mixtures.[14]

Except for the inconstant acute follicular reaction, there is very little evident histological change for the first 36 hours, and the spleen resembles that of a control rat (FIGURE 4A). Mitotic activity then begins in the red pulp. The cells that divide are not the macrophages that ingested the antigen but primitive reticular cells. How do these cells receive their stimulus? They may be stimulated by a digestion product from the macrophage,[18, 19] but at present the question cannot be answered. Mitosis reaches its peak in 3 or 4 days and then gradually subsides. In the meantime, with sufficient antigenic stimulation, the red pulp becomes crowded with large cells that have abundant pyroninophilic cytoplasm and large vesicular nuclei (FIGURE 4B). The spleen weight reaches its peak by day 4, and by this time it may have doubled. The cellular reaction then subsides, and the spleen weight declines. Beginning about day 5 and continuing to about day 8, there is a transient appearance of many small, dark cells adjacent to the few remaining pyroninophilic cells in the red pulp (FIGURE 5A). These cells apparently are derived from the large basophilic cells by cytoplasmic loss and nuclear condensation. They apparently do not break up, but leave the spleen rapidly and have been identified in the blood stream.[17] The serum antibody reaches its peak at about day 6. By 8 or 10 days after the antigenic stimulation the entire spleen resembles that of a control animal. The only noticeable difference is a slight increase in number of mature plasma cells in the red pulp (FIGURE 5B).

Suggested Cellular Reaction Following Two Or more Injections of Antigen

One may assume that the cells or their descendents that proliferated in response to the first injection of antigen migrate into the blood stream and then colonize the lymph node, spleen, bone marrow and possibly many other parts of the body (FIGURE 3B). What are their potentialities and how will they react to a second injection of antigen? From the observations of others it seems likely that lymphocytes from the blood stream have a potential to become macrophages.[20, 21] It is evident from the recent cell transfer studies[22, 23] that lymphoid cells from a previously immunized rabbit can mature into antibody forming cells (presumably plasma cells) with no apparent mitotic activity. Furthermore, in other studies it appears that the cells from a peritoneal exudate rich in macrophages also have the same potentiality.[24, 25]

It is likely that some of the cells released during the primary response return from the blood stream to the spleen and settle out in the follicles as well as the red pulp.[26, 27] Do some of these cells then take part in the secondary reaction? The observations of Leduc *et al.*[28] indicate that germinal center cells may con

tain antibody after secondary stimulation The finding of gamma globulin in germinal centers might be interpreted similarly[24]

The results of experiments reported by Fagraeus[2] suggest that there may be great mitotic activity during the response to repeated antigenic stimulation On the other hand it seems likely from the cell transfer studies[3-5] that mitotic activity *is not necessary for antibody formation during the secondary response*

Relation of Observations to the Clonal Selection Hypothesis

In his recent lectures Burnet[1] has developed the concept first proposed by him in 1957[30] that the self replicating unit that is necessary to explain many of the phenomena of antibody formation is cellular rather than intracellular (enzymatic) Similar suggestions were made independently by Talmage[31] and by members of this laboratory[14] in 1957 The crux of the clonal selection theory as stated by Burnet[1] is that in the animal there exist clones of mesenchymal cells each carrying immunologically reactive sites corresponding in appropriate complementary fashion to one (or possibly a small number of) potential antigenic determinants This provides a population of cells which when an appropriate stage of development has been reached are capable of producing the population of globulin molecules which collectively provide the normal antibodies When an antigen is introduced it will make contact with a cell of the corresponding clone presumably a lymphocyte and by so doing stimulate it to produce in one way or another more globulin molecules of the cell s characteristic type The obvious way of achieving this is to postulate that stimulation initiates proliferation as soon as the cell in question is taken into an appropriate tissue niche spleen lymph node or subacute inflammatory accumulation

As suggested above many of the cells that proliferate in response to a primary antigenic stimulus do not mature chiefly into plasma cells but instead enter the blood stream[17, 18] or the lymph stream[33] as small or medium sized lymphocytes Evidence suggests that these cells would be able to colonize many tissues including the lymph follicles of the organ in which they originally were formed[26] This migration provides a large widely dispersed population of cells with the probable potential capacity to form antibody The prompt participation of these cells in antibody formation would explain the heightened and more generalized response when antigen is injected for the second time and this reaction is in accord with Burnet s clonal selection theory These findings may also explain why the lymph follicles have antibody containing cells after multiple injections of antigen but not after a single injection If one accepts the numerous observations that indicate that lymphocytes can mature into macrophages (phagocytes)[20, 21] then it is apparent that some of these previously stimulated cells may ingest the antigen after the second injection The question whether lymphocytes as well as macrophages can mature directly into plasma cells is still in doubt but some recent evidence provides additional support for this concept[23] Either the prompter processing of the antigen by these prepared macrophages or the direct maturation of these cells into plasma cells could explain the greatly shortened lag period in the secondary response The preservation of clones of lymphocytes as poten

tial antibody forming cells resulting from previous antigenic stimulation gives a cellular foundation for the anamnestic reaction. Plasma cells are rarely seen in the circulation of mammals, and there is general agreement that they do not have the capacity to differentiate into other cell types. These facts make it unlikely that they could carry immunological information from one part of the body to another or that they could function to preserve this information for future accelerated and augmented reactions to a repeated stimulus.

Consideration of Special Problems in Studying the Cellular Reaction to Antigenic Stimulation

At the outset of this discussion the assertion was made that a particulate antigen delivered rapidly to the spleen would result in a brief, localized stimulus. The cellular reaction following this type of stimulus would be expected to be a clear cut and orderly sequence. The alternative reactions produced by other common methods of immunization may be considered. When a soluble antigen is given intravenously it circulates for a considerable period of time and is only gradually localized in the antibody forming tissue. This continued circulation of antigen is likely to result in a prolonged stimulus to the antibody forming tissue lasting several days. Assuming the same sequence of cellular events as has been observed in response to a particulate antigen it is not difficult to visualize the result. There would be a prompt development of antibody forming cells followed by their transformation into small lymphocytes which would be released into the circulation and hence returned to the red and white pulp within a period of 1 week. There also might be continuou stimulation during subsequent days by additional antigen from the blood or lymph. Under these conditions one might expect a rather prolonged primary type of cellular response mixed with a superimposed secondary response. This would be expected to be difficult to interpret histologically. Furthermore the soluble antigen probably would be distributed much more widely among tissues (as well as more widely within a given tissue) than the particulate antigen thus it would be much more difficult to establish a definite histological relationship between antigen localization and cellular response. These and other considerations may help to explain the relatively greater (and later) participation of the follicles of the rabbit spleen following intravenous injection of soluble antigen.[34]

The reaction in regional lymph nodes following subcutaneous antigen injection is probably equally complex. Not only is the antigen delivered to the lymph node over a considerable period of time but inflammation at the site of injection may contribute antibody forming cells that mature and migrate to the lymph node in time to set up a secondary cellular response while the prolonged primary response is still in progress. This type of problem may have influenced the results reported by McNeil.[3, 35] The reaction of the lymph node or the spleen to a homograft a tumor or an infection may be similar since the cellular response may be expected to be prolonged by continuously elaborated antigens. Furthermore local inflammation may supply previously stimulated cells to the lymph node or spleen thus superimposing a secondary type of reaction upon a primary reaction.

An entirely different problem that has received little attention is that of species differences in cellular response. Do the rat,[14] the fowl,[25] the rabbit,[23,24] and man[6] all have essentially the same cellular reaction, or do they exhibit differing histological responses to the same stimulus? Some observations of the cellular pattern correlated with antibody formation exist in each of these species, but they are very difficult to compare because of differences in experimental design including the antigen used and the tissues studied. Preliminary observations in this laboratory made by one of us (F W F) comparing the histological reaction of the rat spleen and the rabbit spleen to the same particulate bacterial antigenic stimulus suggest some similarities and some differences. The comparison of histological response to the same soluble antigen may prove to be much more informative, since the rat fabricates little antibody following injection of any one of many purified proteins commonly used as antigens, even after multiple stimuli.[27]

New Approaches Needed for Old Problems

A number of inadequately studied but important problems need careful investigation. Four of them are listed below, along with suggested approaches.

(1) Comparative histological studies of various species subjected to similar antigenic stimuli should be undertaken. This type of study could do much to increase knowledge of how cells form antibody. Of even greater interest, the information gained might suggest rational explanations of the fundamental differences in immune phenomena among laboratory animals such as the hamster, rabbit, mouse, guinea pig and rat.

(2) Further careful comparison of the histological aspects of the primary and secondary responses in the same species and to the same antigen should be undertaken. In particular, note should be taken of the quantitative tissue distribution of labeled antigen, its radioautographic and fluorescent localization, and its rate of digestion. These observations could be correlated with the histological patterns of cell transformation, including enumeration of the cells containing antibody, the rate of release of antibody into the blood stream, the quantitative aspects of mitotic activity, and the migration of cells from the antibody forming site. The very young animal and the germ free animal might be utilized in some of these studies to ensure that the initial response is as primary as possible within the framework of the natural selection hypothesis.[1,31,33]

(3) Cell transfer studies into immunologically inert animals[3] can probably be utilized to furnish even more information about cell transformations and the ultimate fate of the transferred cells. In similar fashion, survival cultures[19] and clonal tissue culture techniques[41] can be exploited more vigorously in an attempt to simplify the milieu and to compare the cellular transformations after primary and secondary stimulation. Furthermore, the use of tritium labeled thymidine during the proliferative phase of the primary and secondary responses may help to trace and quantitate the transformation of activated reticular cells into lymphocytes, the entry of newly proliferated cells into the blood stream, and their recolonization of lymphoid tissues.

(4) The new techniques that make it possible to test the quantity and

specificity of antibody formed by single cells[41] can contribute greatly to our understanding of the potentiality of cells. While at present there is some confusion as to whether many cell types[4] or, chiefly, the plasma cells[42] produce antibody and whether single cells produce antibody to 1 or more antigens simultaneously[4, 43] these problems will probably resolve themselves with more time and work. This imaginative technique seems destined to extend our knowledge of the cellular aspects of mammalian protein synthesis in general and antibody formation in particular.

These are but a few of the problems and suggested approaches to them. With continued ingenuity and effort, new methods and new avenues should present themselves that will make it possible to obtain additional direct evidence of how cells form antibody.

Summary

The evidence implicating several cell types in the antibody forming mechanism has been reviewed. Mesenchymal cell interrelationships have been outlined. An effort has been made to indicate how the recorded observations of cellular progressions during primary and secondary immune reactions fit into this scheme of cell organization. In particular, the histological patterns observed in the rat spleen after a single particulate antigen injection have been compared with those in the spleen and elsewhere after multiple antigenic stimuli. The relation of these observations to Burnet's clonal selection theory is emphasized. The cellular sequence of events is discussed in the light of the possible implications of the physical state of the antigen, the route of injection, the presence of reproducing antigens (bacterial and cellular), and the species studied. Some of the problems that need special consideration and some new approaches that might help in this study are suggested.

Acknowledgments

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References

1 BURNET, M. 1959. The clonal selection theory of acquired immunity. Vanderbilt Univ. Press. Nashville, Tenn.

2 FAGRAEUS, A. 1948. Antibody production in relation to the development of plasma cells. Acta Med. Scand. Suppl. 204.

3 TALIAFERRO, W. H. 1949. The cellular basis of immunity. Ann. Rev. Microbiol. 3: 159–194.

4 McMASTER, P. D. 1953. Sites of antibody formation. *In* The Nature and Significance of the Antibody Response: 13–45. A. M. Pappenheimer, Jr., Ed. Columbia Univ. Press. New York, N. Y.

5 HARRIS, T. N. & S. HARRIS. 1956. The genesis of antibody. Am. J. Med. 20: 114–132.

6 GOOD, R. A. 1957. Morphological basis of the immune response and hypersensitivity. *In* Host-parasite Relationships in Living Cells. H. M. Felton, Ed. Thomas. Springfield, Ill.

7 MARSHALL, A. H. E. 19 6. An outline of the cytology and pathology of the reticular tissue. Oliver and Boyd. Edinburgh, Scotland.

8 Fitch F W P Barker K H Soules & R W Wissler 1953 A study of the antigen localization and degradation and the histologic reaction in the spleen of normal X irradiated and spleen shielded rats J Lab Clin Med 42 598–620
9 Fitch F W I W Wissler M La Via & I Barker 1956 The timing of antigen injection relative to whole body X irradiation and the development of circulating antibody and the splenic histologic reaction in the rat J Immunol 76 151 160
10 La Via M F P A Barker & R W Wissler 1956 A study of the correlation of antigen phagocytosis and the splenic histological reaction with antibody formation in protein-depleted rats J Lab Clin Med 48 237 254
11 Wissler R W L F Frazier K H Soules P Barker & I C Bristow III 1956 The acute effects of β-3 thienylalanine in the adult male albino rat Arch Pathol 62 62 73
12 Moeschlin S R Pelaez F Hugentobler R Baguena J Baguena & B Demiral 1951 Experimental investigations of the relationship between plasma cells and antibody formation (phase contrast microscope ACTH and cortisone) Compt rend III Congr Soc Intern Europ Hematol I M I S Rome Italy
13 Moeschlin S K Baguena & J Baguena 1953 The influence of ACTH and cortisone on experimental antibody production in rabbits Intern Arch Allergy Appl Immunol 4 83–100
14 Wissler R W F W Fitch M F La Via & C H Gunderson 1957 The cellular basis for antibody formation J Cellular Comp Physiol Suppl 1 50 265–301
15 Rowley D A 1950 The effect of splenectomy on the formation of circulating antibody in the adult male albino rat J Immunol 64 289–295
16 Wissler R W M J Robson F Fitch W Nelson & L O Jacobson 1953 The effects of spleen shielding and subsequent splenectomy upon antibody formation in rats receiving total body X irradiation J Immunol 70 379–385
17 Gunderson C H D Juras M F La Via & R W Wissler Tissue and cellular changes associated with antibody formation in the rat spleen In preparation
18 Lapresle C 1955 Étude de la dégradation de la serumalbumine humaine par un extrait de la rate de lapin II Mise en évidence de trois groupements spécifiques différents dans le motif antigènique de l albumine humain et de trois anticorps correspondants dans le serum de lapin anti albumin humain Ann inst Pasteur 89 654–665
19 Fishman M 1959 Antibody formation in tissue culture Nature 183 1700
20 Maximow A A & W Bloom 1957 Textbook of Histology Saunders Philadelphia Pa
21 Loosli C G 1942 The histogenesis of cells in experimental pneumonia in the dog J Exptl Med 75 657–672
22 Roberts J C & F J Dixon 1955 The transfer of lymph node cells in the study of the immune response to foreign proteins J Exptl Med 102 379–392
23 Neil, A. L & F J Dixon 1959 Immunohistochemical detection of antibody in cell transfer studies A M A Arch Pathol 67 643–649
24 Dixon F J W O Weigle & J C Roberts 1957 Comparison of antibody responses associated with the transfer of rabbit lymph node periton al exudate and thymus cells J Immunol 78 56–62
25 Roberts J C F J Dixon & W O Weigle 1957 Antibody producing lymph node cells and peritoneal exudate cells Morphologic studies of transfers to immunologically inert rabbits A M A Arch Pathol 64 324–332
26 Farr R S 1951 Experiments on the fate of the lymphocyte Anat Record 109 515 534
27 Gowans J L & J M Yoffey et al 1959 The Kinetics of Cellular Proliferation F Stohlman Ed Grune and Stratton New York N Y
28 Leduc A H A H Coons & J M Connolly 1955 Studies on antibody production II The primary and secondary responses in the popliteal lymph node of the rabbit J Exptl Med 102 61–72
29 Ortega L G & R C Mellors 1957 Cellular sites of formation of gamma globulin J Exptl Med 106 627–640
30 Burnet F M 1957–1958 A modification of Jerne s theory of antibody production using the concept of clonal selection Australian J Sci 20 67–69
31 Talmage D W 1957 Allergy and immunology Ann Rev Med 8 239–256
32 McNeil, C 1948 Cellular changes in rabbits during antibody formation I Response to *Eberthella typhosa* Am J Pathol 24 1271–2387
33 Ehrich W E & T N Harris 1942 The formation of antibodies in the popliteal lymph node in rabbits J Exptl Med 76 335–348

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References

1 BURNET M 1959 The clonal selection theory of acquired immunity Vanderbilt Univ Press Nashville Tenn
2 FAGRAEUS A 1948 Antibody production in relation to the development of plasma cells Acta Med Scand Suppl 204
3 TALIAFERRO W H 1949 The cellular basis of immunity Ann Rev Microbiol 3 159–194
4 McMASTER P D 1953 Sites of antibody formation *In* The Nature and Significance of the Antibody Response 13–45 A M Pappenheimer Jr Ed Columbia Univ Press New York N Y
5 HARRIS T N & S HARRIS 1956 The genesis of antibody Am J Med 20 114–132
6 GOOD R A 1957 Morphological basis of the immune response and hypersensitivity *In* Host parasite Relationships in Living Cells H M Felton Ed Thomas Springfield Ill
7 MARSHALL A H E 1956 An outline of the cytology and pathology of the reticular tissue Oliver and Boyd Edinburgh Scotland

THE RETICULOENDOTHELIAL SYSTEM IN EXPERIMENTAL MALARIA AND TRYPANOSOMIASIS

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Although they represent two different taxonomic groups of protozoa, the causative agents of malaria and trypanosomiasis share the biological characteristics of being insect transmitted and producing systemic infections. The malarial parasites are intracellular during most of their lives in the vertebrate host, occurring most commonly in erythrocytes and only temporarily free in the plasma during passage from one infected cell to another. The trypanosomes, on the other hand, spend most of their lives free in the blood plasma and only a few species are normally found within host cells during any part of their cycles. When intracellular they are usually found not in cells of the circulating blood but rather in the tissues of other systems.

Many of the other protozoan pathogens of warm blooded animals (TABLE 1) are parasites of the alimentary tract and its diverticula, and little or nothing is known of their possible connections with the reticuloendothelial system (RES). Some genera of the family Trypanosomatidae are typically intracellular. Infections with the members of the genus *Leishmania* are characterized by parasitism and proliferation in the RES. Parasites of the genus *Endotrypanum* are inhabitants of the red blood cells. The intracellular reproductive stages of most strains of *Trypanosoma cruzi* are encountered in cells of the RES at times, and some strains (which have been designated as reticulotropic) are particularly well adapted to life in this system.[1,2] The importance of the stage of parasite is also important in malarial infections, in which the pre-erythrocytic stages develop in macrophages, splenic reticular cells, and endothelial cells.[3-6] Some of the sporozoa most closely related to the Plasmodiidae are also typically erythrocyte dwellers, while others multiply in endothelial cells or in leukocytes. It is our intention to consider here only infections caused by members of the genera *Plasmodium* and *Trypanosoma*.

By narrowing our field to these two genera, however, we have not simplified our problem, because each contains a number of species that behave in a number of different ways: some have great host specificity, others do not; some produce acute infections, some subacute, others chronic and relapsing. It is almost as impossible to generalize as it would be to particularize in the space available. We have felt that, in addition to presenting our own work, some review of the field might be desirable, and in the treatment of this material we have considered the various species of parasites by groups. The division of the malarial species into avian, simian, and rodent groups is more arbitrary than the division of the trypanosomes into the taxonomic groups of C. A. Hoare, but both methods of division serve the purpose of placing the probably more closely related forms together and of indicating the heterogeneity of the genera.

34 WARD P A A G JOHNSON & M R ABELL 1959 Studies on the adjuvant action of bacterial endotoxins on antibody formation III Histologic response of the rabbit spleen to a single injection of a purified protein antigen J Exptl Med 109 463-474
35 McNEIL C 1950 Cellular changes in rabbits during antibody formation II Multiple antigen injections J Immunol 65 359-367
36 MAKINODAN T P I RUTH & H R WOLFE 1954 Precipitin production in chickens X Cellular changes in the spleen during antibody production J Immunol 72 39-44
37 KENTON H B 1941 The Arthus phenomenon in the rat J Infectious Diseases 69 238-242
38 JERNE N K 19 5 The natural selection theory of antibody formation Proc Natl Acad Sci U S 41 849 857
39 LA VIA M F S A URIU & L A FERGUSON 1960 Studies on antibody production by spleen explants maintained *in vitro* J Immunol 84(1) 48-53
40 MARCUS I I S J CIECIURA & T T PUCK 1956 Clonal growth *in vitro* of epithelial cells from normal human ti ues J Exptl Med 104 615-628
41 NOSSAL G J V & J LEDERBERG 1958 Antibody production by singl cells Nature 181 1419-14 0
42 LENNOX E S & M COHN 1959 Antibody formation by single cells Science 130 1427
43 NOSSAL G J V 1959 Cellular phenotypes and genotypes in antibody formation Science 130 1477

Herman and Goldfarb[19] were able, by means of splenectomy to obtain transient infections in chicks with *P circumflexum* an organism that is normally a parasite of passerine birds only Causey[20] however, reported that splenectomy of canaries had no effect on infections with *P cathemerium* or *P relictum* Terzian[21] found that splenectomy produced a marked lowering of acquired resistance in chickens with *P gallinaceum* but that there was little if any effect on innate resistance Taliaferro and Taliaferro[22] indicate similar results with *P gallinaceum* and *P lophurae* in chicks treated with nitrogen mustard

Anma[23] however found shorter incubation periods and higher mortality in malaria infected birds blockaded with trypan blue and Trager[4] was able to produce higher initial parasitemias with *P lophurae* in chicks following intraperitoneal injection of carbon ink More recently McGhee[25] has had similar results with the same species in ducklings using subcutaneous injections of fat free red blood cell stromata By the use of X irradiation Zain and Wolf[26] were able to increase the number of exoerythrocytic forms of *P gallinaceum* in chicks and Taliaferro *et al* [27] as well as Bennison and Coatney[28] also used this method to obtain higher parasitemias and lower survival times in chicks with *P gallinaceum* Rigdon and Rudisell[29] however found lower parasitemias with *P lophurae* in irradiated chicks, and they concluded that this was attributable to the effect of X rays on the parasites Thompson *et al*[30] found that no effects were exerted by radioactive colloidal iron mixtures on infections with *P cathemerium* in canaries or *P lophurae* in ducklings

The action of cortisone appears to be more pronounced on acquired than on innate resistance Redmond[31] first described the activity of cortisone in *P relictum* infections in pigeons and pointed out that the peak in parasite numbers occurred at about the same time in cortisone treated bird as in controls Following the crisis that results in a primary decline in parasitemias, however the cortisone treated birds frequently die while the parasites decline in untreated birds until none is found by days 9 or 10 In *P gallinaceum* infections however cortisone has been reported to be without effect in chicks[32] or doves[33]

Beginning with the work of Kritchewski and Demidowa[34] there have been a number of studies in which depression of the RES has been combined with chemotherapy in order to show the dependence of drug action on the integrity of the system The above authors used trypan blue as a blocking agent and studied its effects on the treatment of *P relictum* in finches with quinine pamaquine and an acridine antimalarial related to quinacrine In the blockaded birds the parasites appeared sooner and deaths were earlier in spite of therapy

Taliaferro *et al*[35-37] studied the role of the spleen and the lymphoid macrophage system in the quinine treatment of *P gallinaceum* in chicks and concluded that 3 antimalarial factors operate independently in the system namely innate immunity acquired immunity and quinine Bennison and Coatney[28] reported that parasite counts of irradiated quinine treated chicks infected with *P gallinaceum* averaged significantly higher than those of unirradiated quinine treated controls Hughes and Tatum[38] found that minimal 'curative or suppressive doses of primaquine for canaries with *P cathemerium*

The procedures that have been used to demonstrate the function of the RES in protozoan infections have developed, of course, in parallel with those used in studies on bacterial and spirochetal infections and on the physiology of the RES in its other roles. The criteria taken as evidence of suppression of RES activity are various. Effects on innate resistance may be manifested by the development of earlier and higher parasitemias (or more rapid and severe disease) in normal hosts or temporary infections in abnormal hosts. Effects on acquired resistance may be evidenced by the occurrence of relapses in animals that apparently were cured.

Depression of the RES in avian malaria. The earliest studies on the suppression of RES activity in avian malaria were those involving the production of relapse, which was induced in canaries spontaneously cured of *P. praecox* (= *relictum*) infections by the injection of foreign blood from another species of bird.[7] Foreign red cells were subsequently used to produce blockade and induce relapse.[8,9] Gingrich has pointed out that massive treatment is required

TABLE 1
PATHOGENIC PROTOZOA OF HOMOIOTHERMS

Class MASTIGOPHORA
- Family Mastigamoebidae—Genus *Histomonas*
- Family Trypanosomatidae—Genera *Trypanosoma* and *Leishmania*
- Family Trichomonadidae—Genus *Trichomonas*
- Family Hexamitidae—Genera *Hexamita* and *Giardia*

Class SARCODINA
- Family Endamoebidae—Genus *Entamoeba*

Class SPOROZOA
- Family Eimeriidae—Genera *Eimeria* and *Isospora*
- Family Plasmodiidae—Genus *Plasmodium*
- Family Babesiidae—Genera *Babesia* and *Theileria*
- Incertae Sedis—Genera *Toxoplasma* and *Hepatozoon*

Class CILIATA
- Family Bursaridae—Genus *Balantidium*

to produce blockade, which probably accounts for the negative results obtained by others[10] who used janus green and India ink. Cataneï[11] was able to induce relapse with *P. relictum* by injecting cured canaries with avian spirochetes (*Borrelia gallinarum*), but Lion[1] has reported that intercurrent infections of *P. gallinaceum* and *Borrelia gallinarum* in chicks do not influence each other.

Relapses also result from other procedures in which it is not entirely clear just how the RES is affected. Among these are ultraviolet light[13] and epinephrine.[14] Gajewski and Tatum[15] have pointed out that both hypoxia and epinephrine have a feature in common, namely, the production of hyperglycemia. They felt that it was probable that the effects produced by altering blood sugar level were attributable to the influence of glucose on phagocytosis.[16,17]

Although Gingrich[9] failed to confirm the work of Kritchewski and Meersohn,[18] who reported that blockade with trypan blue adversely influences innate resistance in avian malaria, a number of studies have furnished information suggesting that natural as well as acquired resistance can be depressed

studied conveniently in small experimental animals The course of infection with this organism in its natural host a species of tree rat (*Thamnomys surdaster*) is benign and self limiting and in mature white rats and in voles (*Microtus guentheri*) it acts similarly [94] Zuckermann and Yoeli[95,96] have shown that in the vole, the spleen is essential in establishing resistance and that its removal affected both the innate and acquired immune mechanisms In young rats *P berghei* infections may be severe and when young rats are splenectomized before infection a more rapid and fatal disease results [97,98] Splenectomy of mature white rats however had little effect on innate resistance but in both mature and young rats splenectomy seriously interferes with the establishment and maintenance of acquired immunity During these investigations and those of others[99] it was noted that in very young rats splenectomy produced a paradoxical effect on innate resistance enhancing athreptic immunity by interfering with the formation of reticulocytes and thus limiting the most desirable substrate for the parasites This effect which can also be produced by cortisone in the rat [100] will be discussed further in relation to mouse malaria

Parasitic relapses as a result of splenectomy in *P berghei* infected rats have been reported by several authors [101-107] Rodhain[108] had a similar experience using the cotton rat (*Sigmodon hispidus*) as host and Van Riel[109] found the fruit bat (*Roussettus leachi*) to be similar to the cotton rat as an experimental host for this parasite

Two other methods of depressing the RES in rat malaria were reported by Hughes and Tatum [110] They found that hypoxia (equivalent to that at 19 000 feet of altitude) or intercurrent infection with *Trypanosoma lewisi* caused infections with *P berghei* to be more severe and often fatal in rats which do not succumb to this disease under ordinary conditions Jackson [111] however did not confirm this effect in mixed infections Intercurrent infections of *P berghei* and *Haemobartonella muris* have also been studied but the results observed with this combination may be attributable to changes in the erythrocytic series as well as to effects on the macrophage system [11]

When studies on *P berghei* are conducted in mice however the findings are somewhat different Although some investigations on splenectomy[113] and cortisone[114,115] seemed to indicate that the infection was enhanced most later work has shown that the effects of procedures that are usually expected to reduce resistance such as splenectomy [116-119] cortisone administration [97,120,121] and X irradiation[122] do in fact produce an opposite effect because of the importance of the spleen as an erythropoietic agent in mice [123] Suppression of splenic activity results in a paucity of reticulocytes that are the substrate of choice for the parasites The deficiency of young red cells results therefore in a lower and slower infection in mice subjected to those procedures that interfere with erythropoiesis as well as RES activity

Our studies on the RES in experimental malaria have been conducted on *P berghei* in CF1 mice infected by the intravenous injection of 1 250 000 parasites per animal [124] Typical of *P berghei* infections in mice is the minor crisis that occurs between days 3 and 5 after inoculation (FIGURE 1) When colloidal thorium saccharated iron or polyvinyl pyrrolidone are given intravenously

in normal air at 860 feet altitude, must be increased approximately tenfold for birds maintained under hypoxic conditions simulating 19 000 feet of altitude (75 mm Hg partial pressure)

It is of course difficult to separate the cellular function of the RES from the humoral factors in the phenomena of resistance The relation of iso-opsonins to phagocytic activity has been studied by a number of workers[39-41] specific agglutinins and opsonins by others[42-44] and diverse humoral factors in phagocytosis by several more[45-50]

Thus far we have noted mainly functional studies, but before leaving avian malaria the basic histological investigations that have led to our present concepts of the importance of the RES in resistance to avian plasmodia should be mentioned As Taliaferro[51] has pointed out MacCallum[5] observed phagocytosis of bird malaria parasites as early as 1898 and Ben Harel[14] concluded in 1923 that the chief destruction of the parasites was by the fixed tissue phagocytes particularly of the spleen There have been many subsequent studies both immunological and histopathological[63-65]

Depression of the RES in monkey malaria It is interesting to note that the use of splenectomy in the monkey antedated its use in avian malaria by thirty years[66-76] This procedure was used for many years prior to the introduction of blockade[77-79] and it has continued to be a useful technique[80-85]

In 1945 Ekzemplarskaya[86] reported that with the use of an antireticular cytotoxic serum prepared by the immunization of rabbits with an emulsion of spleen and brain from rhesus monkeys (*Macaca mulatta*) she was able to produce a more severe malaria (*P inui*) than that which occurred in control monkeys and to induce relapses of latent infections during the postpatent period

The first observations on the effects of cortisone in experimental malaria (*P cynomolgi*) were made by Schmidt and Squires[87] in rhesus monkeys Intensification of the peripheral blood infection during the postcrisis phase was produced during the primary attack and during chronic or latent stages severe relapses could be induced These observations confirm those obtained with splenectomy blockade and antireticular cytotoxic sera indicating that both innate and acquired resistance can be influenced in monkeys by procedures calculated to depress the RES ACTH has been found to increase the incidence of hemoglobinuria in monkeys infected with *P knowlesi* without effecting significant changes in the parasitemic picture[88]

Certain materials that have been used to produce relapse in simian malaria may or may not act directly in the RES Von Berenberg Gossler[66] used heterologous blood and we have considered that this material exerted its effect through blockade Gonder and Rodenwaldt[67] used horse serum and Mulligan and Sinton[89] who used human blood and Sinton and Mulligan[90] using horse serum all in monkeys have attributed the effect to protein shock that may exert an indirect rather than direct effect on the RES Histological studies relating to the importance of the RES in monkey malaria have also been made by Taliaferro and his colleagues[63, 91-93]

Depression of the RES in rodent malaria With the discovery of *P berghei* in 1948 and its subsequent adaptation to laboratory rodents[94] there became available for the first time a mammalian malarial infection that could be

These results with *P berghei* in mice chiefly involve innate resistance A number of workers have used suppressive drugs in this infection to produce chronic or latent infection that could then be challenged to demonstrate ac

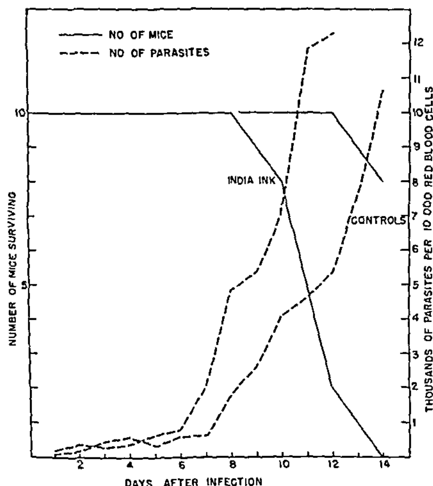


FIGURE 2 Effect of intravenous injection of India ink on *Plasmodium berghei* parasitemia and malarial mortality in CF1 mice Five consecutive daily doses were given the last on the day before infection

quired immunity as noted by Box and Gingrich[15] who used primaquine treatment to eradicate the infection and demonstrated immunity to reinoculation for at least 3 months Most intact animals survived the challenge Splenectomy after cure resulted in malarial mortality in a majority of mice

In contrast to the results that Ekzemplarskaya[56] obtained in monkey malaria using antireticular cytotoxic serum we were unable to affect the course

on 5 consecutive days preceding infection this minor crisis is suppressed in most cases although there is no effect on the survival time in comparison with the controls. This suppression of the crisis in mice treated with thorium dioxide or polyvinyl pyrrolidone results from the failure of the parasites to multiply as rapidly during the first 3 days of infection as they do in the controls. This may be regarded as evidence of an enhancement of innate resistance by these preparations. On the other hand, in mice treated with saccharated iron the crisis is suppressed, even in animals in which parasitemias equaled

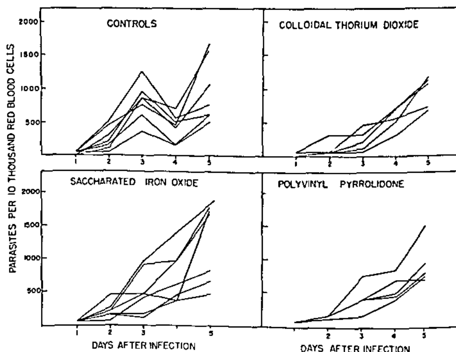


FIGURE 1. Effects of intravenous injection of various colloidal materials on *Plasmodium berghei* parasitemia in CF1 mice. Five consecutive daily doses were given, the last on the day before infection.

those of the controls and this may be interpreted as evidence of interference with the RES and the immune mechanism that effects the drop in parasite count on the fourth day.

When India ink was used (FIGURE 2) according to the same regimen not only was the minor crisis suppressed but higher parasitemias were noted earlier and the survival times were shorter in comparison. When a colloidal suspension of thorium dioxide (Thorotrast) was given on 4 consecutive days beginning on the day before infection a similar pattern was observed. Individual curves are shown in comparison with controls. In each pair the curve at the left represents the parasitemia in a Thorotrast treated animal and the curve at the right represents a control. We believe that these are the only studies done to date on the use of blockade in rodent malaria.

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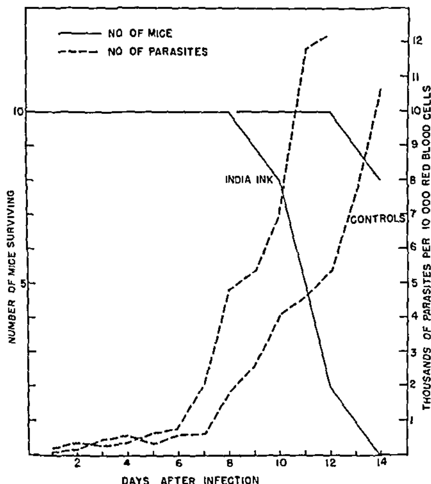


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quired immunity, as noted by Box and Gingrich[125] who used primaquine treatment to eradicate the infection and demonstrated immunity to reinoculation for at least 3 months. Most intact animals survived the challenge. Splenectomy after cure resulted in malarial mortality in a majority of mice.

In contrast to the results that Ekzemplarskaya[88] obtained in monkey malaria using antireticular cytotoxic serum, we were unable to affect the course

of *P berghei* infections in mice with homologous antispleen serum[14] At least part of the failure of such an antiserum to be effective in this host parasite system is probably attributable to the more acute course of the mouse malaria compared with that of the monkey malaria that Ekzemplarskaya employed

Depression of the RES in infections with the Trypanosoma brucei group The

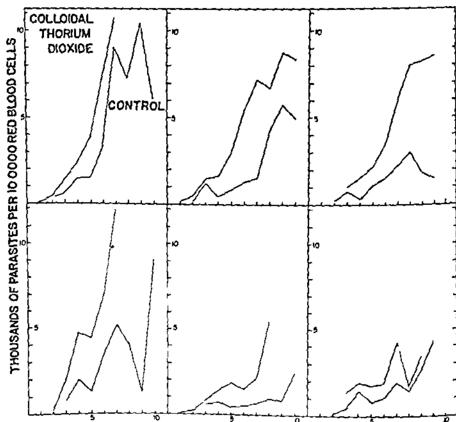


FIGURE 3 Effect of intravenous injection of colloidal thorium dioxide on *Plasmodium berghei* parasitemia in CF1 mice Four consecutive daily doses were given the first on the day before infection

trypanosomes of the *T brucei* group produce in their natural definitive hosts to which they are transmitted by tsetse flies subacute to chronic infections primarily of the blood and blood forming organs but with invasion of other systems in the later stages In rodents to which they can be adapted by artificial transmission they often produce acute infections that provoke no effective immune response

T brucei the causative agent of nagana a disease of cattle and other domestic animals in Africa was studied in relation to splenectomy as early as 1899, at which time Plimmer and Bradford[77] felt that the results in dogs cats

and rabbits were equivocal Later, however they concluded that this procedure in the above mentioned animal as well as in rats did tend to enhance their experimental infection [128] Laveran and Mesnil[129] reported that splenectomy in rats was not effective but Sauerbeck[130, 131] had positive results in rats guinea pigs rabbits and dogs as did Rodet and Vallet[132, 133] in rats Davis[134] reported that splenectomy had no effect on *T brucei* infections in cats

The use of blockade in *T brucei* infections began with the work of Rosenthal and Spitzer[135] and ushered in a period of investigation on the significance of the RES in chemotherapy These authors used saccharated iron while most subsequent investigators usually used India ink [136-139] Von Jancso and von Jancso[140, 141] used an electrocolloidal copper that they considered an RES poison Most of the above authors used blockade in conjunction with splenectomy but Kritchewski[138] and Pockels[139] also did some work along these lines using splenectomy alone All of these investigators concurred in finding that interference with the RES had profound influences on the effects of chemotherapy, and that an intact RES was necessary for optimum drug action

Rosenthal and Spitzer[135] also employed thorium X to the extent of reducing greatly the peripheral lymphocyte counts They found however that this damage did not reduce the ability of the host to respond to drug treatment

As a corollary to the importance of the RES in increasing the efficacy of medication came the recognition by von Jancso and von Jancsó[141] that the condition of the RES was important in relation to the development of drug resistance which could be induced much more readily in animals that were splenectomized or blockaded

Fewer studies have been done with *T gambiense* the causative agent of Gambian sleeping sickness Kritchewski and his colleagues [142, 143] who studied this organism in mice found that splenectomy and/or blockade had little influence on the course of the infection The studies of Tschenkower[144] with splenectomized rats likewise failed to show a significant influence of the spleen in resistance to this parasite Nieschulz and Wawo Roentoe [145] however reported that in splenectomized dogs the infection was greatly enhanced in comparison with controls and Amako[146] indicated that the resistance of mice to this organism could be depressed by blockade Schwetz[147] also reported splenectomy in mice and rats to be effective in depressing the RES

With *T rhodesiense* the causative agent of East African human trypanosomiasis even fewer studies have been done Splenectomy has been reported to exert no effect on the infection in cats [134] Cortisone has been studied by Ashcroft[148, 149] and found to exert different effects on *T rhodesiense* infection in rats depending on dosage and time of administration Under certain conditions the infection was enhanced and under others cortisone was seen to protect the animals from some of the adverse effects of the parasitemia

Depression of the RES in infections with the Trypanosoma evansi group Most of the observations that have been made with organisms of the *T brucei* group have been confirmed by work in the *T evansi* group at a somewhat later time *T evansi* which causes a disease of horses called surra and a comparable disease of camel called suaru was studied as early as 1907 by Laveran and Thiroux [150] as well as by Massaglia [151] These authors and the later in

of *P berghei* infections in mice with homologous anti pleen serum[14] At least part of the failure of such an antiserum to be effective in this host parasite system is probably attributable to the more acute course of the mouse malaria compared with that of the monkey malaria that Fkzemplarskaya employed

Depression of the RLS in infections with the Trypanosoma brucei group The

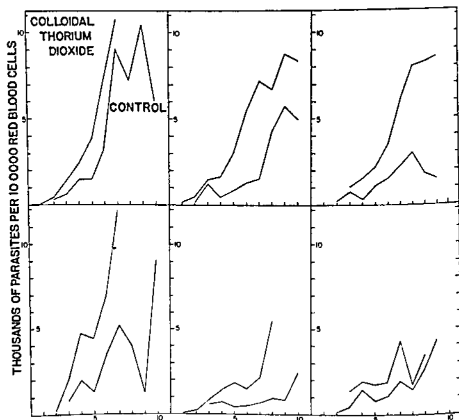


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ferro *et al*[194] in whose experiments the other organism was *Haemobartonella muris* and by Hughes and Tatum[110] with *P berghei* Naiman[193] used X irradiation and Jaroslow[191] did some similar work Hughes and Tatum[195] found that hypoxia effected an increased level of parasitemia and some fatal infection but that acquired immunity in rats that had recovered from a normal course of infection could not be broken down by hypoxic condition

Many of the experiments made on *T lewisi* in rats have also been conducted with *Trypanosoma duttoni* the corresponding species nonpathogenic to mice Splenectomy blockade and X irradiation have been studied by Galliard[196] Taliaferro and Pavlinova[197] and Jaroslow[195] respectively and all three procedures have been used in various combinations by Jaroslow[194]

Depression of the RES in infections with Trypanosoma cruzi As a result of histopathological studies[1 2 199 207] the importance of the RES in infection with *T cruzi* the causative agent of Chagas disease has been recognized for many years Although this parasite occurs in the blood in the trypanosome form it does not multiply there it reproduces by binary fission of intracellular leishmanial forms that inhabit various tissues including heart muscle neuroglia and reticuloendothelial cell Splenectomy has been consistently reported to have no influence on the infection in mice rats guinea pigs or dog[14 145 204 08 13] Although some experiments in the use of blockade[204 14 15] have been inconclusive[214] Denison[17 215] was able by the use of trypan blue to influence the resistance of rats and more recently by the use of intravenous colloidal thorium dioxide prior to inoculation we (unpublished data) have been able to obtain early and high parasitemia in mice with avirulent strains that ordinarily produce only low or late infections Recently Pizzi (personal communication) has obtained even more striking results using X irradiation and producing high parasitemias in rats with a strain that normally produces a low level of infection in this host

Cortisone has been observed by a number of workers to be effective in depressing the RES in *T cruzi* infections In 1951 Wolf *et al*[219 220] found that latent *T cruzi* infections in monkeys were exacerbated by cortisone treatment bringing into patency natural infections that had not been observed or suspected before medication The work of Jarpa and his colleagues[221 224] was simultaneous and independent Subsequently Seneca and Rockenbach[225] reported that fatal infections in rats could be induced by the use of cortisone with strains of *T cruzi* that were normally avirulent and the Chilean workers have extended their work with cortisone in this infection[226-234]

Other steroidal compound however do not produce a depressing effect on experimental Chagas disease Robles *et al*[235] reported that the clinical picture in infected dogs showed striking improvement when ACTH was given Prompted by the observation that the course of infection in male mice was more rapid and more severe than in females one of us (F C G)[236] performed some experiments in which certain sex hormones were tested for possible effects on the course of experimental Chagas disease Testosterone progesterone estrone and diethylstilbestrol were tested in mice of both sexes and no significant differences in mortality rates were observed between treated and control animals of the same age and sex

Depression of the RES in infections with the Trypanosoma congolense group

vestigators[14, 15] all reported that splenectomy had no effect on *T. evansi* infections in rats, mice, guinea pigs, or rabbits. Several investigators have found that splenectomy depresses the RES in rats, mice, and dogs, although rabbits and guinea pigs were not affected.[153-158] On the other hand, it has been reported that latent *T. evansi* infections in rabbits could be induced to relapse by the injection of olive oil.[159] Cortisone does not affect the disease in mice.[160]

The work of Kritschewski[154, 155] was patterned along the previous lines of investigation on the importance of the RES in therapy, and subsequently a number of other authors took up work along these lines using another species, *T. equiperdum*, the parasite that causes dourine in horses. Although Linton[161] and Taliaferro *et al.*[16] found that splenectomy had no effect on infections with this organism in guinea pigs and mice, respectively, subsequent workers[162-173] used splenectomy, blockade, or both, and came to the same conclusion regarding the necessity of the RES for optimum results in treatment as were reached as a result of similar studies on *T. brucei*.

The importance of the conditions of the RES in the development of resistance noted by von Jancso and von Jancso[141] with *T. brucei* was confirmed by the work of Schnitzer *et al.*[174] with *T. equiperdum*.

Cooper[175] found that X irradiation depresses the RES in *T. equiperdum* infections in rats, as evidenced by reduced susceptibilities to tryparsamide, but Kligler and Comaroff[176] reported no effect with *T. evansi* in the same host.

Although some reports on cortisone in *T. equiperdum* infections indicated that there was no influence on the rate of development of the parasites or the survival of the rats or mice,[177-179] other studies[180, 181] showed that the steroid interfered with the action of oxophenarsine hydrochloride at higher doses and had an adverse effect on immunization if the antigen dose was near its threshold.

Depression of the RES in infections with the Trypanosoma lewisi group. *T. lewisi*, the nonpathogenic parasite of rats, is of particular interest because of the studies of Taliaferro[18] who described in *T. lewisi* infections a reaction product (subsequently called ablastin) that inhibits the cell division of the parasites but that does not kill them. Regendanz and Kikuth[183] demonstrated the importance of the role of the RES in the formation of this antibodylike substance, showing that in splenectomized rats division of the parasites continued several days longer than in intact controls and that, in some splenectomized animals, the trypanosomes never ceased to divide and the infection terminated fatally. Further demonstrations of the importance of the spleen for the production of antibodies in *T. lewisi* infection was also furnished by studies of Taliaferro *et al.*,[184] of Regendanz,[185] and of Taliaferro,[186] who used blockade as well as splenectomy. There are also a number of other interesting studies involving splenectomy.[187-190]

Members of the *T. lewisi* group have very high host specificity. By means of splenectomy, however, Brynoghe and Vassiliadis[191] were able to transmit *T. lewisi* of rats to harvest mice, which are normally refractory to infection with this species.

Intercurrent infection has also been shown to depress the RES to a point where *T. lewisi* infections are more severe. This has been studied by Talia

P berghei and West Nile virus in mice Jacobs [51] noted a similar effect with *P lophurae* and ornithosis in chicks and Trager [52] with *P lophurae* and a virus of ducks that produces splenic necrosis On the other hand in other intercurrent infections such as *P gallinaceum* with *Borrelia gallinarum* in chicks the two diseases developed independently [12]

Certain studies in which infection with one organism has had an influence on another must be carefully regarded with a view to possible humoral cross immune reactions which may be operating rather than more specific RES stimulation Such are those involving two strains of the same species of malaria [53] (*P knowlesi* in monkeys) or two species of malaria [38] (*P gallinaceum* and *P lophurae* in chicks) Related to this may be the phenomenon of *occultation parasitaire** observed by Sergent and Poncet [254] with *P relictum* and *P rouxi* in canaries Some avian mixed infections however develop independently as *P cathemerium* and *P lophurae* do in ducks [255]

Stimulation of the RES in trypanosomiasis In 1904 Nissle [256] reported that the intraperitoneal inoculation of *Serratia marcescens* from potato culture into rats infected with *T brucei* resulted in a lowered parasitemia Two years later Massaglia [57] noted that intercurrent infections with streptococci caused trypanosomes to disappear from the blood and in 1907 Schein [58] recorded similar findings in a dog concurrently infected with *T evansi* and *Bacillus anthracis* Subsequently Rodet *et al* [259] reported further on the reciprocal influences of mixed infections with trypanosomes and bacteria Trautman [260] made the first observation on the antagonism between relapsing fever spirochetes (*Borrelia duttoni*) and *T brucei* Further observations on intercurrent infections of spirochetes and trypanosomes of the *T brucei* and *T evansi* groups have continued throughout the years [261-273] The species of trypanosomes that have been shown to be suppressed in mixed infections with spirochetes are in addition to *T brucei* *T gambiense* *T rhodesiense* *T evansi* *T equinum* *T equiperdum* and *T congolense* The species of spirochetes that antagonize the development of the trypanosome infections (presumably by stimulating the RES) are in addition to *B duttoni* *B merionesi* *B microti* and *B crocidurae* Other species such as *B hispanica* and *B persica* do not produce this effect and *B turicatae* has a variable behavior [279, 280]

Spiral organisms of the species (*Spirillum minus*) that causes sodoku a type of rat bite fever also have this effect on infections in guinea pigs with *T brucei* [281] and *T gambiense* [25] as well as in rats with *T equinum* and *T lewisi* [253] It has been suggested that considerable damage to the RES is involved in the spirochete infections [284, 285] and it must be inferred therefore that the effect on the trypanosome infections results from a compensatory hyperactivity of the system in response to this injury Galliard [6] has pointed out that the antagonistic effect is reduced if animals are splenectomized just before or just after infection

As pointed out with reference to mixed malarial infections the evaluation of the effects of intercurrent infections with different species of the same genus is difficult A few studies have been done along these lines [287, 293] but we feel

* Masking of parasitemia

The members of this group are tsetse fly transmitted parasites of African mammals. Although Browning *et al.*[277] reported that splenectomy produced no effect on *T. congolense* infection in mice, Schwetz[147] found that splenectomy acts as a depressant in both mice and rats, and Goble and Boyd[38] were able to enhance infections in mice by the use of thorium dioxide blockade. Goble *et al.*[126] also were able to accelerate infections with *T. congolense* in mice by the use of inhibiting doses of rabbit antimouse spleen serum.

Desowitz and Watson[39] found that splenectomy of rabbits led to consistently lethal infections with *T. simiae*, the causative agent of acute porcine trypanosomiasis, which is not inoculable to most small laboratory animals. Splenectomy of rats, mice, and guinea pigs, however, failed to reduce the refractivity of these animals to the infection.

Depression of the RES in infections with the Trypanosoma vivax group. Although Desowitz and Watson[240] were unable to affect the course of infections in rats with *T. vivax*, a parasite of sheep and cattle that is transmitted to small laboratory animals with extreme difficulty, Unsworth[241] was able to establish this parasite in continuous mouse to mouse passage when splenectomized mice were used.

Stimulation of the RES in malarial infections. The fact that certain agents used in the production of blockade may under certain circumstances stimulate rather than depress the RES has been recognized for some time.[4] Such an effect was noted in *P. gallinaceum* infections in chicks by Schuleman and Knoche,[243,244] who used colloidal palladium. A few authors have suggested the possibility that injections of serum may stimulate nonspecifically the RES in avian malaria. If this occurred, it would represent the converse of the use of serum to depress the RES and cause relapse, as noted previously in connection with simian malaria. The experimental evidence for the stimulatory effect, however, is quite equivocal.[36,245,247]

Although Ekzemplarskaya[86] was able to demonstrate RES stimulation with a homologous antispleen brain serum in monkeys with *P. inui*, which is a benign, chronic, relapsing species, we were unable to find any effect of homologous antispleen serum in mice with *P. berghei*, which pursues a subacute and fatal course.[1,6]

Hegner and Dobler,[248] while attempting to influence infections of *P. cathemerium* and *P. relictum* in canaries by altering the reticulocyte percentages, found that the infections were less severe when the birds were maintained in an atmosphere high in oxygen. These findings were interesting in view of the reports of Gajewski and Tatum[15] and Hughes and Tatum[38] on the depression of resistance by hypoxia. It should be noted that the hypoxic conditions used by these authors to produce relapses and to interfere with the effectiveness of drugs were the equivalent of conditions at 19,000 to 20,000 feet of altitude. At conditions equivalent to a little more than one half that altitude, however, hypoxia produced a stimulating effect on the RES, according to Herbig Sandreuter[49] and to Geigy and Freyvogel.[250]

There are reports that intercurrent infections with certain viral diseases that attack the RES may stimulate this system to the extent that malarial infections are suppressed. Yoeli *et al.*[251] observed this in mixed infection with

nene[299] During the cour e of this work certain findings have led us to digress in order to elucidate their meaning Two areas of collateral investigation may be de cribed briefly

The polyoxyethylene ethers were selected for te ting both on account of

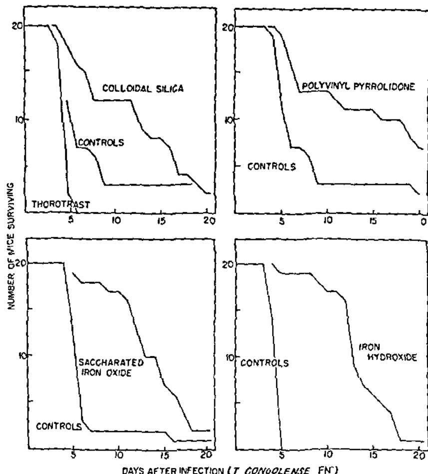


FIGURE 4 Effects of ntravenous injection of ari us c lloidal materials on survival of DBA mice with *T ypano ma c ng lense* infections Four consecutive daily doses were given the first on the day before infection

their macromolecular nature and becau e they had been reported to be active *in vivo* but not *in vitro* against murine tuberculosis[300] It has been postulated that these agents enhance the ability of the monocytes to suppress the multiplication of intracellular tubercle bacilli[301] Although both the linear and cyclic types of polyoxyethylene ethers have activity of the same order in tuberculosis we found that the linear representative (WR 1339) was much more

that much additional work[297, 93] is necessary before a critical appraisal in this area can be attempted

We know of only one other procedure calculated to stimulate the RES that had been attempted in experimental trypanosomiasis up to the time we began our studies along these lines This was the work of Ewing and Emerson[94] who were unable to produce measurable effects in mice infected with *T equiperdum* using homologous antireticular cytotoxic sera As we have pointed out elsewhere[176] the fulminating infection produced by that parasite in mice is by its acute nature a less satisfactory system for the demonstration of an effect in which time is such an important factor Browning *et al*[37] had tried to enhance *T congolense* infections by the use of blockade with saccharated iron and had reported that they found no evidence of marked influence on the animals resistance There is, however in their own data indication that the blocking agent had the reverse of the expected effect in their experiments so that 3/6 blockade mice did not become infected at all In our experiments with *T congolense* we have been able to demonstrate stimulation of the RES with both homologous and heterologous anti spleen and antilymph node sera as well as with certain blocking agents[1, 6, 38]

We have employed *T congolense* for a number of studies[95] in which we wished to observe more subtle effects than could be noted with the more rapidly fatal infections that result with members of the *T brucei* and *T evansi* groups When 500 000 parasites per mouse are used for the infecting dose the median survival time with the Wellcome TN strain of *T congolense* is between 5 and 9 days which allows time for RES depression or stimulation to take effect and to be observed We have established a routine screening method that will show these effects involving the use of 4 consecutive daily intravenous injections of the test materials the first injection being given on the day before infection

From among the preparations that we tested and that had been used for RES blockade in other systems only Thorotrast was found to enhance *T congolense* infections in mice (FIGURE 4) The curve shown in the figure for Thorotrast indicates the effect at a maximum tolerated dose Graded reduction of the dose from this suppressive level did not result in the establishment of a stimulating dose Other materials such as colloidal iron colloidal silica and polyvinyl pyrrolidone were found to exert a protective effect delaying the median survival times as long as 15 days beyond that of the controls

Nearly 100 other colloidal macromolecular and polymeric substances have been screened for similar activity Only a few of them seemed to alter the course of the infection and in all cases the effect was one of prolongation rather than acceleration Among those that were found to be without influence in this system were the bacterial lipopolysaccharides Piromen and zymosan[96] both of which were tested according to numerous regimens that had been reported to be effective in altering the course of bacterial infection in mice The Tween series of emulsifiers also was found to be ineffective Also inactive in this system were certain simpler materials suggested as RES stimulants as a result of other studies namely choline[297] histamine[9] and limo

nene [99] During the cour e of this work certain findings have led us to digress in order to elucidate their meaning Two areas of collateral investigation may be described briefly

The polyoxyethylene ethers were selected for te ting, both on account of

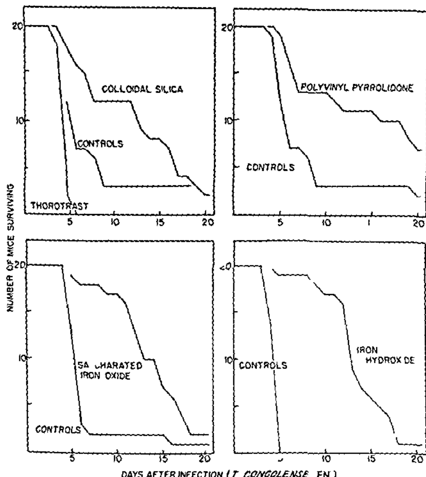


FIGURE 4 Effe ts of intravenous injection of various coll idal materials on su vi al of DBA mice with *Trypan oma cong lense* infections Four consecuti e dail dose ere given the first on the day before infection

their macromolecular nature and becau e they had been reported to be active *in vivo* but not *in vitro* against murine tuberculosis [100] It has been postulated that these agents enhance the ability of the monocytes to uppress the multiplication of intracellular tubercle bacilli [101] Although both the linear nd cyclic types of polyoxyethylene ethers have activity of the same order in tuberculosis we found that th linear r presentative (WR 1339) was much more

that much additional work[87–93] is necessary before a critical appraisal in this area can be attempted.

We know of only one other procedure calculated to stimulate the RES that had been attempted in experimental trypanosomiasis up to the time we began our studies along these lines. This was the work of Ewing and Emerson[234] who were unable to produce measurable effects in mice infected with *T. equiperdum* using homologous antireticular cytotoxic sera. As we have pointed out elsewhere[16] the fulminating infection produced by that parasite in mice is, by its acute nature, a less satisfactory system for the demonstration of an effect in which time is such an important factor. Browning *et al.*[237] had tried to enhance *T. congolense* infections by the use of blockade with saccharated iron and had reported that they found "no evidence of marked influence on the animals' resistance." There is, however, in their own data, indication that the blocking agent had the reverse of the expected effect in their experiments, so that 3/6 blockade mice did not become infected at all. In our experiments with *T. congolense* we have been able to demonstrate stimulation of the RES with both homologous and heterologous antispleen and antilymph node sera, as well as with certain blocking agents.[16, 238]

We have employed *T. congolense* for a number of studies[95] in which we wished to observe more subtle effects than could be noted with the more rapidly fatal infections that result with members of the *T. brucei* and *T. evansi* groups. When 500,000 parasites per mouse are used for the infecting dose, the median survival time with the Wellcome TV strain of *T. congolense* is between 5 and 9 days, which allows time for RES depression or stimulation to take effect and to be observed. We have established a routine screening method that will show these effects, involving the use of 4 consecutive daily intravenous injections of the test materials, the first injection being given on the day before infection.

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Nearly 100 other colloidal, micromolecular, and polymeric substances have been screened for similar activity. Only a few of them seemed to alter the course of the infection, and in all cases the effect was one of prolongation rather than acceleration. Among those that were found to be without influence in this system were the bacterial lipopolysaccharides Piromen and zymosan[236] both of which were tested according to numerous regimens that had been reported to be effective in altering the course of bacterial infection in mice. The Tween series of emulsifiers also was found to be ineffective. Also inactive in this system were certain simpler materials suggested as RES stimulants as a result of other studies, namely, choline,[97] histamine,[98] and limo

in the RES in mice[303] the studies of Berry and Mitchell[304] and of Rogers[305] seemed to indicate, as Bohme *et al*[306] have noted that differences in host susceptibility were not explicable by differences in the initial clearance mechanism These authors however have shown that in mice infected with *Salmonella typhimurium* two different strains are different in the kinetics of their response to infection in terms of carbon clearance and that the difference was not in magnitude of the increase in RES activity following infection, but rather in time of onset

The CFW mice used in our experiments are presumably relatives of the Swiss Webster strain which has been repeatedly studied by Benacerraf[7,308] and others and found to demonstrate RES adequacy in clearance tests It seems possible therefore that the differences in response to micromolecular materials that we have observed in infected mice also may be a function of time and that further experimentation with different regimens may resolve this disturbing and devious problem

Conclusion

The principal functions of the RES in relation to the hemoflagellate and Hemosporidea were recognized by Linton[161] in 1929 and reaffirmed by Taliaferro[309] in the same year They recognized that certain protozoan parasitoses were primarily infections of the RES that in malaria the chief defense was probably phagocytosis by the RES that the RES was responsible for the formation of humoral antibodies and that the RES was necessary in mediating the curative action of certain drugs Our understanding of all of these aspects has been greatly amplified by studies during the past thirty years Many new details and approaches have been added without the emergence of many new concepts The three most noteworthy of the new ideas we believe relate to (1) the importance of the RES in the development of drug resistance (2) the recognition of the lymphoid macrophage system and the importance of mesenchymal reserves as set forth by Taliaferro[31,59,65,97,310-313] on the basis of numerous careful histopathological studies by himself and his colleagues and (3) the possibility that the RES may be stimulated by the use of certain chemical and biological agents some of which may be quite nonspecific

Although we have been unable to consider in detail many of the informative and imaginative studies that have been or are being pursued it is our hope that by viewing the field broadly we have succeeded in suggesting the usefulness and interest that the blood protozoa have as tools for the study of the RES

References

1 BADINEZ O 1945 Bi l gica Santiago 3 3 52
2 TALIAFERRO W H & T Pizzi 1955 J Infectious Diseases 96 199 276
3 MANWELL R D & F GOLDSTEIN 1939 Science 89(2302) 131 132
4 COULSTON F 1949 Proc Soc Exptl Biol Med 70 360-364
5 HUFF C G 1954 J Infectious Diseases 94 173 1/
6 CORRADETTI A 1955 Trans Roy Soc Trop Med Hyg 49(4) 311 333
7 MOLDOVAN J 1912 Centr Bakteriol Orig 66(1) 10 110
8 WHITMORE E R 1918 J Hopkins Hosp Bull 29(32) 62-67
9 GINGRICH W D 1941 J Infectious Diseases 68(1) 3 -45

active than the cyclic one (HOC 12½) in trypanosomiasis It was then necessary to investigate the *in vitro* activity of these compounds against trypanosomes We found thereby that the linear type was active against the parasites in the test tube It was apparent, therefore that only a part of its

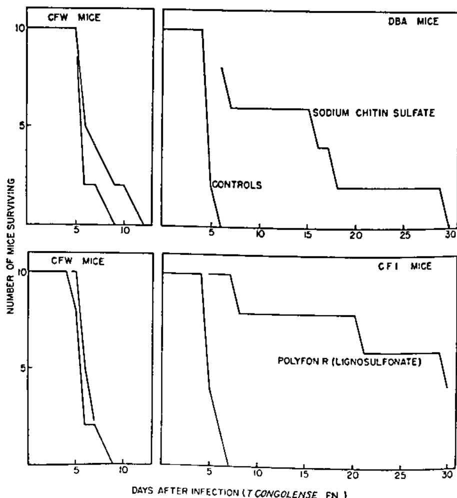


FIGURE 5 Effects of intravenous injection of certain macromolecular materials on the survival of different strains of mice with *Trypanosoma congolense* infections Four consecutive daily doses were given the first on the day before infection

activity could be attributable to RES stimulation The cyclic form however was without activity *in vitro* and even at low dilutions had a slight *in vivo* effect which is considered to be the result of its action on the host [30]

Another finding which has resulted in ancillary studies that are still under way related to the strain difference in response to certain polymeric substances (FIGURE 5) Although histological studies have revealed strain differences

63 TALIAFERRO W H & W BLOOM 1945 J Infectious Diseases 77(2) 109 138
64 HERBBERGER I K & C K COATNEY 1946 Am J Path 22 3) 654-6.5
65 TALIAFERRO W H & L C TALIAFERRO 1955 J Infectious Diseases 97(2) 99 142
66 VON BERENBERG G W LER H 1909 Arch Troti tenk 16(3) 24 280
6 GONDER R & F KODENWALDT 1910 Centralbl Bakt I Abt Orig 54(3) 236-240
68 LECER M & M BOUILLIEZ 1912 Compt rend soc biol 73 28) 310 313
69 BOUILLIEZ M 1913 Compt rend soc biol 74(18) 10 0 1072
0 BLANCHARD R & M LANGERON 1913 Arch Parasitol 15(4) 599-60
71 NOGUCHI H 1928 J Exptl Med 47 821 827
72 BRUMPT E 1929 J Egypt Med Assoc 12(3) 31 3
3 BRUMPT E 1929 Arch Anatom Microscop 25 49 55
4 KRISHNA K V, K O V SMITH & C LAL 1933 Indian J Med Research 21(?) 343 35?
5 KRISHNA K V K O V SMITH & C LAL 1934 Indian J Med Research 21(3) 639-64
6 KNOWLES R & B M DAS GUPTA 1934 Indian Med Gaz 69 10) 541 54
MALAMOS B 1934 Arch Schiffs u Tropen Hyg 38 8) 326-342
8 MAUCK E G & B MALAMOS 1935 Z Immunität forsch 84(4) 33 3 8
79 MAUCK E G & B MALAMOS 1936 Arch Schiff Tropen Hyg 40(5) 18 19
80 SINTON J A 1935 Rec ls Malaria Surve India 4(4) 501 71
81 CASSUTO N 1936 Riv Malari I 15 n s 11(4) 240 257
82 MULLIGAN H W T SOMMERVILLE & C S SWAMINATH 1940 J Malaria Inst India 3(4) 91-601
83 MAIER J & L T COGGESHALL 1944 J Exptl Med 79(4) 401-430
84 WOLFSON F & M W WINTER 1946 Am J Hyg 44(2) 273-300
85 BRAY R S 19 9 Trans Roy Soc Trop Med Hyg 53 3) 300
86 FAZEMPLAR KAYA F V 1945 Med Parazitol Parazitar Bolezni 14(1) 34 37
87 SCHMIDT C H & W L SQUIRES 1951 J Exptl Med 94(6) 501 20
88 KASS F H Q M GEIMAN S H INGBAR A B LEY J W HARRI & M FINLAND 1951 Proc 2nd ACTH Conference 2 3 6-350
89 MULLIGAN H W & J A SINTON 1933 Records Malaria Surv y India 3(3) 579-568
90 SINTON J A & H W MULLIGAN 1933 Records Malaria Survey India 3 4) 719 67
91 TALIAFERRO W H 1932 Am J Hyg 2 4 9-449
92 TALIAFERRO W H & P K CANNON 1936 J Infectious Diseases 59 1) 2 125
93 TALIAFERRO W H & C KELVER 1940 J Infectious Diseases 67(2) 167 1 6
94 VINCKE I H & M LIPS 1948 Ann soc belge med trop 28(1) 97 104
95 ZUCKERMANN A & M YOELI 1951 J Infectious Diseases 89(2) 130-142
96 ZUCKERMANN A 1953 J Infectious Diseases 92 3) 205-223
97 RAMAKRISHNAN S P S PRAKASH & A K KRISHNASWAMI 1951 Indian J Malariol 5(3) 447-454
98 ZUCKERMANN A & M YOELI 1954 J Infectious Diseases 94(3) 225-236
99 GALLIARD H & J LAPIERRE 1951 Bull soc pathol exotique 44(3-4) 185-194
100 JACKSON G J 1955 J Infectious Diseases 97(2) 157 159
101 GALLIARD H 1949 Bull soc pathol exotique 42 9/10) 431-433
102 GALLIARD H & J LAPIERRE 1950 Compt rend soc biol 5/6 402 403
103 CORRADETTI A 1950 Riv Parassitol 11(4) 203-209
104 BLACK R H 1951 Ann Trop Med Parasitol 45(3/4) 199-206
105 FABIANI G R VARGUES P GRELLET & J CLAUSE 1951 Compt rend soc biol 145(15/16) 1131 1134
106 FABIANI G & G FULCHIRON 1954 Compt rend soc biol 148(5/6) 530-533
107 FABIANI C & G FULCHIRON 1954 Compt rend soc biol 148 6/3-6 5
108 RODHAIN J 1949 Ann soc belge med trop 39(4) 483-489
109 VAN RIEL J 19 0 Ann inst Pasteur 79(5) 172 6
110 HUGHES F W & A L TATUM 19 6 J Infectious Diseases 99(1) 38-43
111 JACKSON G J 1959 J Parasitol 45(1) 94
112 YIN MING H C D & Q M GEIMAN 1952 Am J Trop Med Hyg 1(5) 747 760
113 MATTILA V J APARICIO GARRIDO L PRIETO & FERNANDEZ NAFRIA 1954 Med colonial Madrid 23 8 11
114 FINDLAY G W M & F M HOWARD 1952 Nature 169(4300) 547
115 GALLIARD H J LAPIERRE M LARIVIÈRE & R BERDONNEAU 1953 Compt rend acad sci 236(12) 1308 1310
116 SINGER I 1954 J Infectious Diseases 94(2) 159-163

10 SCHUURMAN C J & A M SCHUURMAN TEN BOKKEL HUININK 1929 Mededeel Dienst Volksgezondheid Ned Indie Foreign Edition 18(3) 469-497
11 CATANEI A 1925 Arch inst Pasteur Algerie 3 111 121
12 LION M 1939 Bull Soc Pathol Exotique 32(7) 713-716
13 WHITMORE E R 1927 J Trop Med 2 475-485
14 BEN HAREL S 1923 Am J Hyg 3(6) 652-685
15 CAJEWSKI J F & A I TATUM 1944 J Infectious Diseases 74(2) 85 92
16 HEGNER R W & M S MACDOUGALL 1926 Am J Hyg 6(4) 602-609
17 MACDOUGALL M 1927 Am J Hyg 7(5) 635-647
18 KRITSCHEWSKI I L & J S MEERSOHN 1932 Z Immunitätsforsch Exptl Therapy 76(6) 499-506
19 HERMAN C M & A I GOLDFARB 1939 Am J Trop Med 19(6) 595-598
20 CAUSEY O R 1939 Am J Hyg 30(3) 93 99
21 TERZIAN L A 1946 J Infectious Diseases 79(3) 215-220
22 TALIAFERRO W H & L G TALIAFERRO 1948 J Infectious Diseases 82(1) 15 30
23 ARIMA J 1934 Fukuoka Acta Med 27(1) 12-13
24 TRAGER W 1941 Am J Hyg 34(3) 141-149
25 MCGHEE R B 1959 J Parasitol 45(4 section 2) 48
26 ZAIN H & A WOLF 1943 Deut Tropenmed Z 47(3) 68-71
27 TALIAFERRO W H L G TALIAFERRO & E L SIMMONS 1945 J Infectious Diseases 77(2) 158 176
28 BENNISON B E & G P COATNEY 1945 Public Health Repts 60(5) 127-132
29 RIGDON R H & H FUDISELL 1945 Proc Soc Exptl Biol Med 59(2) 167 1 0
30 THOMPSON P E D A MCGINTY D L BUSH & M I WILSON 1948 J Infectious Diseases 83(1) 23 32
31 REDMOND W B 1952 Proc Soc Exptl Biol Med 79 258-261
32 SCHNEIDER J 1953 Bull Soc Pathol Exotique 46(6) 1016-1023
33 GRIGNASCHI V J 1954 Exptl Parasitol 3(1) 30-37
34 KRITSCHEWSKI I L & I W DEMIDOWA 1934 Z Immunitatsf Exptl Therapy 84(1) 14 21
35 TALIAFERRO W H 1948 J Infectious Diseases 83 164-180
36 TALIAFERRO W H & F F KELLEY 1948 J Infectious Diseases 83(2) 181-199
37 TALIAFERRO W H & L G TALIAFERRO 1949 J Infectious Diseases 84(2) 187 220
38 HUGHF F W & A I TATUM 1955 J Infectious Diseases 97(3) 231 237
39 ZUCKERMAN A 1945 J Infectious Diseases 77(1) 28-59
40 COFFIN G S 1951 J Infectious Diseases 89(1) 8-15
41 SCHWINK T M 1954 Am J Trop Med Hyg 3(2) 232-249
42 TRAGER W L STAUBER & S BEN HAREL 1950 Proc Soc Exptl Biol Med 75(3) 766-771
43 STAUBER L A H A WALKER & A P RICHARDSON 1951 J Infectious Diseases 89(1) 31-34
44 BEN HAREL S 1951 Am J Hyg 53(1) 1 10
45 TALIAFERRO W H & L G TALIAFERRO 1940 J Infectious Diseases 66(2) 153 165
46 TALIAFERRO W H & L G TALIAFERRO 1945 J Infectious Diseases 77(3) 224-248
47 TALIFERRO W H & L G TALIAFERRO [illegible] J Inf[illegible]tious Di[illegible]ases 86)3) 275 294
48 TRAGER W & R B MCGHEE [illegible] 91(4) 365 379
49 ALVAREZ D A JR 1952 [illegible] J Hyg 66 [illegible] 31 38
50 BECKER E P T M S[illegible] & R I [illegible] Iowa State Coll J Sci 27 [illegible]0-90
51 TALIAFERRO W H 1949 [illegible]
52 MACCALLUM W C 1898 J Exptl M[illegible] 3 [illegible] 10[illegible] 11[illegible]
53 TALIAFERRO W H & L G TALIAFERR[illegible] M[illegible] 3(3) 197-208
54 TALIAFERRO W H & L G T[illegible] M[illegible] 3(3) 209 223
55 MULLIGAN H W 1929 Ind[illegible] J M[illegible] R[illegible] 16 [illegible] 1106
56 MULLIGAN H W 1929 Indian J M[illegible] R[illegible] 16 [illegible]
57 CANNON P R & W H TALIAFERR[illegible] 1931 J Pr[illegible] M[illegible] 5(1) 3 64
58 BRUMPT E 1936 Ann parasit hum[illegible] 14 [illegible] 670
59 TALIAFERRO W H & H W MULLIGAN [illegible] Indian M[illegible] R[illegible] Memoirs 29 1 138
60 MULLIGAN H W T SOMMERVILLE & C S SWAMINATH[illegible] 1940 J Malaria Inst India 2(4) 563-579
61 TREZIAN L A 1941 Am J Hyg (C) 33 [illegible] 33 53
62 RIGDON R H 1944 Am J Trop Med 24 [illegible] 37[illegible]

168 REINER L C S LEONARD & S S CHAO 1932 Arch intern pharmacol therapy 43(1) 37–48
169 REINER L & S S CHAO 1932 Arch intern pharmacol therapy 43(2) 209 215
170 PFEIFFER C C & A L TATUM 1935 J Pharmacol Exptl Therap 53(3) 358 376
171 BUNTING C H A L TATUM & C C PFEIFFER 1935 Trans Assoc Am Physicians 50 253 256
172 ZOLOG M & O COMSIA 1936 Compt rend soc biol 122(24) 1135 1137
173 ZOLOG M & O COMSIA 1936 Compt rend soc biol 122(24) 1138 1140
174 SCHNITZER R J L C LAFFERTY & M BUCK 1946 J Immunol 54(1) 47 57
175 COOPER C A 1930 J Pharmacol Exptl Therap 39(3) 255 256
176 KLIGLER I J & R COMAROFF 1935 Am J Hyg 22(1) 11–17
177 VON BRAND T F J TOBIE & B MEHLMAN 1951 Am J Hyg 54(1) 76–81
178 AGOLINI C C CAVICCHINI & C GRASSI 1953 Boll soc ital biol sper 28(8 10) 1571 1573
179 CANTRELL W 1959 J Infectious Diseases 104 71 77
180 CANTRELL W 1955 J Infectious Diseases 96(3) 259 267
181 CANTRELL W & G D BETTS 1956 J Infectious Diseases 99(3) 282 296
182 TALIAFERRO W H 1924 J Exptl Med 29(1) 171 190
183 REGENDANZ P & W KIKUTH 1927 Centr Bakteriol Parasitenk 1 Abt Orig 103(4/5) 271 279
184 TALIAFERRO W H P R CANNON & S GOODLOE 1931 Am J Hyg 14(1) 1 37
185 REGENDANZ P 1932 Z Immunitätsforsch 76(6) 437–445
186 TALIAFERRO W H 1938 J Infectious Diseases 62(1) 98 111
187 PERLA D & J MARMORSTON GOTTESMAN 1930 J Exptl Med 52(4) 601–616
188 SCHWETZ J 1931 Ann parasitol humaine et comparée 9(1) 10–14
189 SCHWETZ J 1934 Bull soc pathol exotique 27(1) 62 70
190 PERLA D 1939 Proc Soc Exptl Biol Med 40(1) 91–94
191 BRUYNOGHE R & I C VASSILIADIS 1929 Ann soc belge med trop 9(2) 191–195
192 VASSILIADIS P 1930 Arch Intern Med Exptl 6(1) 89 118
193 NAIMAN D N 1944 J Parasitol 30(4) 209–229
194 JAROSLOW B N 1959 J Infectious Diseases 104 119 129
195 HUGHES F W & A L TATUM 1956 J Infectious Diseases 98 127 132
196 GALLIARD H 1933 Bull soc pathol exotique 26(4) 609–613
197 TALIAFERRO W H & A PAVLINOVA 1936 J Parasitol 22(1) 29–41
198 JAROSLOW B N 1955 J Infectious Diseases 96(3) 242 249
199 SOUZA CAMPOS F DE 1927 Bol Biol S Paulo 16 99 107
200 SOUZA CAMPOS L DE 1927 Ann fac med univ S Paulo 4 75 90
201 SOUZA CAMPOS F DE & I DE TOLEDO ARTIGAS 1932 Ann fac med univ S Paulo 7 95 115
202 DIAS E 1932 Compt rend soc biol 110(18) 206–210
203 SOUZA CAMPOS F DE 1934 Rev biol hyg S Paulo 5(1) 48–49
204 DIAS E 1934 Mem inst Oswaldo Cruz 28(1) 1 110
205 MAZZA S & M E JORG 1935 Novena Reun Soc Argent Patol Region 1 122 231
206 MAZZA B & M E JORG 1935 Novena Reun Soc Argent Patol Region 1 365–411
207 MAZZA B & M E JORG 1943 Mission Estud Pat Reg Argent (Univ B A) 65 3 58
208 GALLIARD H 1930 Bull soc Pathol exotique 23(2) 188 192
209 REGENDANZ P 1930 Zentr Bakteriol Parasitenk 1 Abt Orig 116(4/5) 256–264
210 KOFOID C A & F DONAT 1933 Bull soc path exotique 26(2) 8 9
211 WOOD F D 1934 *Am J Trop Med 14(6) 497–517*
212 KELSER R A 1936 Am J Trop Med 16(4) 405–415
213 PIZZI T & F KNIEREM 1955 Bol Chileno Parasitol 10(3) 42–49
214 PIZZI T 1944 Tesis Facultad de Medicina Univ Chile Santiago Chile
215 FRIEBEL H V 1952 Arch Exptl pathol Pharmakol Naunyn Schmiedebergs 216(6) 536–40
216 PIZZI T 1957 Monografia Biol Univ Chile 7 183 Santiago Chile
217 DENISON N 1943 Proc Soc Exptl Biol Med 52(1) 26–27
218 DENISON N 1943 Am J Hyg 38(2) 178–184
219 WOLF A E A KABAT A E BEZER & J R C FONSECA 1951 Federation Proc 10(1) 26–27
220 WOLF A E A KABAT A E BEZER & J R C FONSECA 1953 *In* The Effect

117 Fabiani G & J Orfila 1955 Compt rend soc biol 149(1/2) 87-90
118 Galliard H & J Lapierre 1957 Congr Natl Sci Med Rep Pop Roumaine Bucarest
119 Lapierre J M Sretenovic & J J Rousset 1958 Bull soc pathol exotique 51(6) 897-901
120 Roberts O J 1954 Parasitology 44(1/2) 58 64
121 Singer I 1954a J Infectious Diseases 94(2) 164 172
122 Singer I 1953 J Infectious Diseases 92(1) 97-104
123 Singer I 1954c J Infectious Diseases 94(3) 241-261
124 Singer I 1954b J Infectious Diseases 94(3) 237-240
125 Box E D & W D Gingrich 1958 J Infectious Diseases 103 291-300
126 Coble F C J L Boyd & I Singer 1958 J Immunol 80(3) 204 214
127 Plimmer H G & J R Bradford 1899 Nature 60(1552) 309-311
128 Bradford J R & H G Plimmer 1902 Quart J Microscop Sci n s 45 449 471
129 Laveran A & F Mesnil 1902 Ann inst Pasteur 16(1) 1-55
130 Sauerbeck E 1905 Z Hyg Infecktionskrankh 52(1) 31 86
131 Sauerbeck E 1906 Z Hyg Infektionskrankh 53(3) 512 514
132 Rodet A & C Vallet 1906 Compt rend acad sci 142(22) 1229 1231
133 Rodet A & G Vallet 1907 Compt rend acad sci 145(4) 281 283
134 Davis L J 1931 Ann Trop Med Parasitol 25 79-90
135 Rosenthal F & E Spitzer 1924 Z Immunitätsforsch 40(6) 529 551
136 Kritschewski I L 1927 Centr Bakteriol Abt Orig 104 214-218
137 Jungeblut C W 1927 Z Hyg Infektionskrankh 107 357-379
138 Feldt A & A Schott 1927 Z Hyg Infektionskrankh 107 453-471
139 Lockels W 1934 Arb staatsinst exptl Therap u Georg Speyer Haus Frankfurt a M 29 12-45
140 von Jancsó M & H von Jancsó 1934 Ann Trop Med 28(3) 419-438
141 von Jancsó M & H von Jancsó 1935 Ann Trop Med Parasitol 29(1) 95-109
142 Kritschewski I L & L Schwartzmann 1928 Z Immunitatsforsch 56 322-329
143 Kritschewski J L O M Semzova & M I Ratner 1930 Z Immunitatsforsch 67(5/6) 417-434
144 Tscherikower R Z 1930 Z Immunitatsforsch Exptl Therapy 68(1/2) 182 192
145 Nieschulz O & F K Wawo-Roentoe 1930 Z Immunitätsforsch 65 312
146 Amako T H 1930 Zentr Bakteriol Parasitenk Abt I 116 280
147 Schwetz J 1934 Bull soc pathol exotique 27(3) 253-260
148 Ashcroft M T 1957 Ann Trop Med Parasitol 51(3) 301-312
149 Ashcroft M T 1959 J Infectious Diseases 104 130-137
150 Laveran A & A Thiroux 1907 Compt rend acad sci 145(1) 14 18
151 Massaglia M A 1907 Compt rend acad sci 145 572
152 Knowles R & B M Das Gupta 1928 Indian J Med Research 15(4) 997-1057
153 Witkamp J 1925 Ned Ind Bladen u Diergeneeskd 37(4) 385-394
154 Kritschewski I L 1927 Z Immunitatforsch 53 506-531
155 Kritschewski I L 1928 Z Immunitatsforsch 59(1) 1-16
156 Kligler I J 1929 Ann Trop Med Parasitol 23(3) 315-324
157 Nieschulz O & F K Wawo Roentoe 1930 Tijdschr Diergenesk 56(21) 1133-1136
158 Nieschulz O & F K Wawo Roentoe 1930 Z Infektionskrankh parasit krankh u Hyg Haustiere 38(1) 14-22
159 Kligler I J & I Weitzman 1926 Proc Soc Exptl Biol Med 23 355-358
160 Frieble H A 1952 Arch Exptl Pathol u Pharmacol 216(6) 515-535
161 Linton R W 1929 Arch Pathol 8(3) 488-501
162 Taliaferro W H T L Johnson & I R Cannon 1929 *In* The Immunology of Parasitic Infections W H Taliaferro Ed 239 Century New York N Y
163 Kelly H M 1930 J Pharmacol Exptl Therap 39(3) 256 257
164 Cooper W A 1930 J Pharmacol 39(3) 257
165 Reiner L C S Leonhard & S S Chao 1930 Proc Soc Exptl Biol Med 27 791
166 Reiner L & C S Leonhard 1930 Proc Soc Exptl Biol Med 27 798
167 Benn V A & G A Cooper 1931 J Pharmacol Exptl Therap 42(3) 254-255

168 REINER L C S LEONARD & S S CHAO 1932 Arch intern pharmacol therapy 43(1) 37–48
169 REINER L & S S CHAO 1932 Arch intern pharmacol therapy 43(2) 209 215
170 PFEIFFER C C & A L TATUM 1935 J Pharmacol Exptl Therap 53(3) 358 376
171 BUNTING C H A L TATUM & C C PFEIFFER 1935 Trans Assoc Am Physicians 50 253 256
172 ZOLOG M & O COMSIA 1936 Compt rend soc biol 122(24) 1135 1137
173 ZOLOG M & O COMSIA 1936 Compt rend soc biol 122(24) 1138 1140
174 SCHNITZER R J L C LAFFERTY & M BUCK 1946 J Immunol 54(1) 47 57
175 COOPER G A 1930 J Pharmacol Exptl Therap 39(3) 255–256
176 KLIGLER I J & R COMAROFF 1935 Am J Hyg 22(1) 11–17
177 VON BRAND T F J TOBIE & B MEHLMAN 1951 Am J Hyg 54(1) 76–81
178 AGOLINI C C CAVICCHINI & C GRASSI 1953 Boll soc ital biol sper 28(8 10) 1571 1573
179 CANTRELL W 1959 J Infectious Diseases 104 71 77
180 CANTRELL W 1955 J Infectious Diseases 96(3) 259–267
181 CANTRELL W & C D BETTS 1956 J Infectious Diseases 99(3) 282 296
182 TALIAFERRO W H 1924 J Exptl Med 29(1) 171 190
183 REGENDANZ P & W KIKUTH 1927 Centr Bakteriol Parasitenk I Abt Orig 103(4/5) 271 279
184 TALIAFERRO W H L R CANNON & S GOODLOE 1931 Am J Hyg 14(1) 1 37
185 REGENDANZ P 1932 Z Immunitätsforsch 76(6) 437–445
186 TALIAFERRO W H 1938 J Infectious Diseases 62(1) 98 111
187 PERLA D & J MARMORSTON GOTTESMANN 1930 J Exptl Med 52(4) 601–616
188 SCHWETZ J 1931 Ann parasitol humaine et comparée 9(1) 10–14
189 SCHWETZ J 1934 Bull soc pathol exotique 27(1) 67 70
190 PERLA D 1939 Proc Soc Exptl Biol Med 40(1) 91–94
191 BRUYNOGHE R & P C VASSILIADIS 1929 Ann soc belge med trop 9(2) 191 195
192 VASSILIADIS P 1930 Arch Intern Med Exptl 6(1) 89–118
193 NAIMAN D N 1944 J Parasitol 30(4) 209–228
194 JAROSLOW B N 1959 J Infectious Diseases 104 119 129
195 HUGHES F W & A L TATUM 1956 J Infectious Diseases 98 12 132
196 GALLIARD H 1933 Bull soc pathol exotique 26(4) 609–613
197 TALIAFERRO W H & A PAVLINOVA 1936 J Parasitol 22(1) 29–41
198 JAROSLOW B N 1955 J Infectious Diseases 96(3) 242 249
199 SOUZA CAMPOS E DE 1929 Bol Biol S Paulo 16 99 102
200 SOUZA CAMPOS E DE 1929 Ann fac med univ S Paulo 4 75–90
201 SOUZA CAMPOS E DE & P DE TOLEDO ARTIGAS 1932 Ann fac med univ S Paulo 7 95 115
202 DIAS E 1932 Compt rend soc biol 110(18) 206–210
203 SOUZA CAMPOS E DE 1934 Rev biol hyg S Paul 5(1) 48–49
204 DIAS E 1934 Mem inst Oswaldo Cruz 28(1) 1 110
205 MAZZA S & M E JÖRG 1935 Novena Reun Soc Argent Patol Region 1 122 231
206 MAZZA S & M E JORG 1935 Novena Reun Soc Argent Patol Region 1 365 411
207 MAZZA S & M E JORG 1943 Mission Estud Pat Reg Argent (Univ B A) 65 3 58
208 GALLIARD H 1930 Bull soc Pathol exotique 23(2) 188 192
209 REGENDANZ P 1930 Zentr Bakteriol Parasitenk 1 Abt Orig 115(4/5) 256–264
210 KOFOID C A & F DONAT 1933 Bull soc path exotique 26(2) 8 9
211 WOOD F D 1934 Am J Trop Med 14(6) 497 517
212 KELSER R A 1936 Am J Trop Med 16(4) 405–415
213 PIZZI T & F KNIEREM 1955 Bol Chileno Parasitol 10(3) 42–49
214 PIZZI T 1944 Tesis Facultad de Medicina Univ Chile Santiago Chile
215 FRIEBEL H A 1953 Arch Exptl pathol Pharmakol Naunyn Schmiedeberg's 218(6) 536–40
216 PIZZI T 1957 Monografia Biol Univ Chile 7 183 Santiago Chile
217 DENISON N 1943 Proc Soc Exptl Biol Med 52(1) 26–27
218 DENISON N 1943 Am J Hyg 38(2) 178 184
219 WOLF A E A KABAT A E BEZER & J R C FONSECA 1951 Federation Proc 10(1) 26–27
220 WOLF A E A KABAT A E BEZER & J R C FONSECA 1953 *In* The Effect

of ACTH and Cortisone Upon Infection and Resistance G Schwartzman Ed 122–139 Columbia Univ Press New York N Y
221 Jarpa A M Agosin R Christen & A V Atias 1951 Bol inform parasit Chilenas 6(2) 25 27
222 Neghme A M Agosin R Christen A Jarpa & A V Atias 1951 Bol Inform parasit Chilenas 6(3) 36
223 Agosin M R Christen & A Jarpa 1951 J Parasitol 37(5 sect 2) 31
224 Agosin M R Christen A Jarpa & A Atias 1951 Biol Santiago 14/15 29–54
225 Seneca H & J Rockenbach 1952 Science 116(1) 14–15
226 Neghme A O Badinez A Jarpa F Thiermann M Agosin & R Christen Bol inform parasit Chilenas 7(1) 4–6
227 Agosin M R Christen A Jarpa V A Atias & A Neghme 1952 Rev Med Chile 80(1) 34–37
228 Pizzi T 1952 Bol inform parasit Chilenas 7(2) 25–27
229 Pizzi T M Rubio R Prager & R Silva 1952 Bol inform parasit Chilenas 2 22 24
230 Thiermann F & R Christen 1952 Bol inform parasit Chilenas 7(4) 53 55
231 Pizzi T M Rubio & F Knierim 1954 Parasitology 15(4) 577–592
232 Rubio M 1954 Biol Santiago 20 89 125
233 Rubio M 1955 Biol Santiago 21 75–83
234 Pizzi T & J Chemke 1955 Biol Santiago 21 31–58
235 Robles G J M Perrin & J Balcazar 1951 Proc 2nd Clin ACTH Conference 1 468–477
236 Goble F C 1952 J Parasitol 38(4 sect 2) 15
237 Browning C H D F Cappell & R Culbransen 1934 J Pathol Bacteriol 39 (1) 65–74
238 Goble F C & J L Boyd 1957 J Protozool Suppl 4 17
239 Desowitz R S & H J C Watson 1953 Ann Trop Med Parasitol 47(3) 324–334
240 Desowitz R S & H J C Watson 1953 Ann Trop Med Parasitol 47(1) 62–67
241 Unsworth K 1953 Ann Trop Med Parasitol 47(3) 232–239
242 Jaffe P H 1931 Physiol Revs 11(3) 277–327
243 Schulemann W & E Knoche 1941 Arch exptl pathol Pharmakol Naunyn Schmiedeberg s 197(3) 227–239
244 Knoche E 1941 Arch exptl pathol Pharmakol Naunyn Schmiedeberg s 197(3) 240–251
245 Maxwell R D & F Goldstein 1941 J Exptl Med 71(3) 409–422
246 Russell P F & B N Mohan 1942 J Exptl Med 76(5) 477–495
247 Russell P F B N Mohan & P Putman 1943 J Parasitol 29(3) 208 216
248 Hegner R W & M Dobler 1941 Am J Hyg 34(1) 14–17
249 Herbig Sandreuter A 1953 Acta Trop 10(1) 1–27
250 Geigy R & T Freyvogel 1954 Acta Trop 11(2) 167–171
251 Yoeli M Y Becker & H Bernkopf 1955 Harefuah 49(6) 116–119
252 Jacobs H R 1957 Proc Soc Exptl Biol Med 95(2) 372–373
253 Trager W 1959 Proc Soc Exptl Biol Med 101(3) 578–582
254 Sergent E & A Poncet 1952 Arch inst Pasteur Algerie 30(1) 31–43
255 Wolfson F 1945 Am J Hyg 41(1) 123 135
256 Nissle A 1904 Hyg Rundschau 14(21) 1039–1041
257 Massaglia A 1906 Boll Acad Med Geneva 21(1) 15
258 Schein H E F 1907 Ann inst Pasteur 21(9) 39–757
259 Rodet A Rubinstein & Bader 1910 Bull soc pathol exotique 3(2) 83 87
260 Trautmann R 1907 Ann inst Pasteur 21(10) 808 824
261 Daels F 1910 Arch Hyg 72(4) 257 306
262 Savini E 1923 Compt rend soc biol 88(12) 956–958
263 Vinzent R 1926 Compt rend soc biol 95(23) 286 288
264 Vinzent R 1927 Ann inst Pasteur 41(2) 131 14
265 Gwelessiany S 1927 Bull soc pathol exotique 20(7) 653–664
266 Delorme M & T E Anderson 1928 Compt rend soc biol 98(14) 1183 1185
267 Galliard H 1928 Bull soc pathol exotique 21(4) 315 316
268 Galliard H 1929 Ann parasitol humaine et comparee 7(1) 57 60
269 Tanzer F 1930 Arch ital sci med colon 11 91 103
270 Kawamura H 1931 Zentr Bakteriol Parasitenk 1 Abt Orig 120 59–78
271 Velu H L Balozet & G Zottner 1931 Compt rend soc biol 106(12) 1092 1094

272 Thompson J C & P de Muro 1932 J Trop Med Hyg 35(3) 33 36
273 Grillo J 1933 Zentr Bakteri I Parasitenk Orig 128(3/4) 252 265
274 Stroder J 1936 Z Immunitätsforsch 89(3/4) 161 177
275 Guo K D 1937 Zentr Bakteriol Parasitenk I Abt Orig 139(3/4) 113 125
276 Vaisman A 1947 Bull soc pathol exotique 40(3/4) 74 6
277 Nocra M 1954 Bull soc path exotique 47(6) 788 790
278 Lapierre J M Lariviere & J J Rousset 1958 Bull soc pathol exotique
61(2) 173 176
279 Galliard H J Lapierre & J J Rousset 1957 Bull soc pathol exotique
50(5) 663–666
280 Galliard H J Lapierre & J J Rousset 1958 Ann parasitol humaine et
Comparee 33(3) 177 208
281 Grillo J & R Krumeich 1934 Zentr Bakteri I Parasitenk 1 Abt Orig 132
(7/8) 30 -403
282 Ceccaldi J & F Guilhaumon 1942 Rev sci Med Pharm Vet Afr Fr 1(1)
16–38
283 Tate P 1951 Parasitology 41(1/2) 117 127
284 Hassko A 1931 Zentr Bakteriol Parasitenk I Abt Orig 123 140 150
285 Schue berger H J & J Grillo 1935 Zentr Bakteriol Parasitenk 1 Abt Orig
135(4/5) 203 215
286 Galliard H 1931 Compt rend soc biol 107(24) 1282 1284
287 Laveran A 1916 Bull soc pathol exotique 9(9) 731–738
288 Schilling C 1930 J Trop Med 33(22) 334 336
289 Tseng H 1935 Zentr Bakteriol Parasitenk 1 Abt Orig 134(3/4) 153 159
290 Galliard H 1934 Ann Parasitol 12(4) 273 277
291 Grillo J & J Schmitz 1935 Z Immunitätsforsch 85(3/4) 203 217
292 Schilling C 1936 Z Immunitätsforsch 87(5/6) 492 518
293 Roubaud E 1951 Bull soc pathol exotique 44(5/6) 343 3 0
294 Ewing I L & G A Emerson 1946 Federation Proc 6(1) 177
295 Goble F C & J L Boyd 1959 Proc Soc Exptl Biol Med 100(4) 745 750
296 Goble F C & J L Boyd 1957 J Protozool Suppl 4 17
297 Heller J H 1957 *In* Physiopathology of the Reticulo Endothelial System B
N Halpern Ed 43–49 Blackwell Scientific Publ Oxford and Masson et Cie
Paris France
298 Gozsy B & L Kátó 1957 Arch intern pharmacodynamie 112(1/2) 235 246
299 Kátó L & B Gozsy 1958 Arch intern pharmacodynamie 117(1/2) 52–67
300 Hart P D A & R J I Rees 1955 CIBA Foundation Symposium on Experi
mental Tuberculosis 299 308 Churchill London England
301 Mackaness G B 1954 Am Rev Tuberc 69(5) 690–704
302 Goble F C J L Boyd & J D Fulton To be published
303 Dunn T B 1954 Natl Cancer Inst 14(6) 1281 1433
304 Berry J L & R B Mitchell 1953 Texas Repts Biol Med 11 379
305 Rogers D F 1958 The Pasteur Fermentation Centennial A Scientific Sympo
sium 61 Chas Pfizer New York N Y
306 Bohme D H H A Schneider & J M Lee 1959 J Exptl Med 110(1) 9 26
307 Benacerraf B M Sebestyen & S Schlossman 1959 J Exptl Med 110(1)
27–48
308 Benacerraf B M Sebestyen & N S Cooper 1959 J Immunol 82(2) 131 137
309 Taliaferro W H 1929 The Immunology of Parasitic Infections 414 pp Cen
tury New York N Y
310 Taliaferro W H 1934 J Parasitol 20(3) 149 161
311 Taliaferro W H & L G Taliaferro 1951 J Immunol 66(2) 181 212
312 Taliaferro W H 1956 Am J Trop Med Hyg 5(3) 391 410

THE RETICULOENDOTHELIAL SYSTEM AND NONSPECIFIC RESISTANCE

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In 1886 Wyssokowitsch working in the newly established laboratory of H Flügge at Gottingen Germany observed for the first time bacteria in the phagocytic cells lining the sinusoids of the liver of a rabbit[1] In his report which appeared as the first article in the first issue of the *Zeitschrift für Hygiene* Wyssokowitsch postulated this mechanism to be of greater importance for bacterial clearance than phagocytosis by the neutrophils of the circulating blood, which recently had been described by Elie Metchnikoff Since then the discussion as to the relative importance of these two cell types among the factors of resistance against infectious disease has never ceased completely

It was soon understood that the original concept, that phagocytosis equals the death of the ingested microorganism was fallacious, and that the bactericidal or bacteriostatic power of the neutrophil depended upon the bacterial species ingested and its degree of virulence The monocyte however which had been included by K Kiyono[2] in the reticuloendothelial system (RES) under the name of blood histiocyte because of its ability to take up vital dyes, was for a long while, and frequently still is conceived as the corner stone of nonspecific resistance This opinion prevailed until Maximow and Doan and Sabin[3] demonstrated, in 1927 that tubercle bacilli are able to multiply inside monocytes This observation was abundantly confirmed[4, 5] and was recently found to be true also for *Salmonella typhimurium*[6] Under the influence of these studies the pendulum swung back and the macrophage was considered more as a liability than as an asset at least in the case of an infection with the tubercle bacillus[7] More recently however exhaustive investigations have demonstrated that the phagocytic and bacteriostatic properties of monocytes like those of microphages are influenced by at least 3 independent factors namely the state of immunity of the host[4, 8] the virulence of the bacterial species involved[6] and the presence of antibodies in the serum in which the monocyte is suspended[9, 10] Unexpectedly however the role of these antibodies appeared to be rather nonspecific

Unfortunately present knowledge is far less advanced with regard to the phagocytic and bacteriostatic properties of the fixed reticuloendothelial cells in liver and spleen The origin of the phagocytic cells remains under debate until this very day Two principal theories have been formulated the first considers the Kupffer's cell as a specialized part of the vascular endothelium[11] while the second regards it as derived from the circulating monocyte The latter is believed to descend to the liver sinusoid in rhythmic time intervals[12] after having been filtered out of the blood stream[13, 14] The more recent investigations favor the second view but undisputable proof still remains to be furnished

Although the mode of action of the reticuloendothelial cells in spleen and lymph nodes has received a great deal of attention comparatively little is

known about the metabolic function of the reticuloendothelial apparatus in the liver. In 1929, Nikolaus von Jancsó demonstrated that the Kupffer's cells of the rat liver ingest particulate matter in two steps: during the first stage the particle is adsorbed to the cell surface and during the second one ingested into the cytoplasm, provided that the serum with which the liver is perfused is kept at normal body temperature.[13] The kinetics of this reaction were worked out subsequently by Halpern *et al.*[14-16] who measured the disappearance of colloidal carbon of known particle size from the blood stream. Biozzi recently reported that a heat-denatured complex of albumin and globulin tagged with I^{131} and administered by vein was broken down in the Kupffer's cells of the mouse liver in accordance with the Law of Michaelis and Menten. Preceding injection of colloidal carbon decreased the rate of breakdown in keeping with a competitive inhibition mechanism. These results were interpreted to indicate the operation of enzymatic processes in the Kupffer's cell.[17, 18] These results are in good correlation with the histochemical demonstration of acid phosphatase in the cytoplasm of the Kupffer's cell (Wachstein and Meisel;[19] Wachstein, personal communication).

By far the most widely investigated function of the RES concerns its place among the mechanisms of defense against infection. The evidence for an active role of the RES in nonspecific resistance has been based chiefly upon two different experimental approaches: (1) the demonstration in the infected animal of an increased ability to clear intravenously injected particulate matter from the blood stream, and (2) the impairment of the defense of an experimental animal through the blockade of its RES. In spite of the most ingenious experimental techniques, neither method has provided an unequivocal answer to date. The first approach is beset by the difficulty of deciding how far ingestion of bacteria is followed by their destruction. This question cannot be answered with certainty until it is possible to examine the phagocytic and bacteriostatic properties of isolated Kupffer's cells. The first experiments undertaken in this direction by Rous and Beard in 1933 yielded only inconclusive results, since the cells that these authors isolated from dog livers failed to ingest any particulate matter in the isolated state.[20, 21]

The second approach, which has enjoyed and continues to enjoy a great amount of popularity, has not yielded fully unequivocal results. Even if we disregard the contradictory reports of those authors who claim to have blocked the RES by removal of the spleen, a substantial body of evidence remains where investigators have employed intravenous injection of saccharated iron oxide, trypan blue, thorium dioxide, or colloidal copper in addition to extirpation of the spleen and have arrived at diametrically opposed results. With the blocking technique it was found, for instance, that the RES possessed a definite protective function against *Borrelia recurrentis*[22, 23] and avian malaria,[24] while it had no effect upon the outcome of an infection with *Bacillus pseudoanthracis*,[25] *Mycobacterium tuberculosis*[26] or *Spirocheta (Borrelia) duttoni*.[27] The most recent investigation, on the other hand, furnished good evidence for a protective role of the RES in hemorrhagic[28] and traumatic shock.[29]

Part of the difficulties encountered in analyzing the results obtained with this technique arise from the fact that its mechanism of action is understood

incompletely. To date it is as yet unknown whether a blocking injection of, for instance, colloidal thorium dioxide (Thorotrast) really reaches all reticuloendothelial cells accessible at the time of injection, whether blocking implies a truly physical saturation of the cytoplasm or the cell surface, whether the resistance-depressing effect is really mediated by the RES or whether specific pharmacological properties of the blocking agent come into play that act independently of the RES. There are comparatively few data available about the pharmacological effects of these agents. In the case of thorium dioxide it appears that administration of this compound results in a disturbance of the oxidative capacity of the Kuffer's cells and changes in the electrostatic proper-

TABLE 1

EFFECT OF INTRAPERITONEAL INJECTION OF *SALMONELLA ABORTUS EQUI* ENDOTOXIN (PYREXAL) AND ACETONE-WASHED BCG ON SUSCEPTIBILITY OF MICE TO INFECTION WITH *MYCOBACTERIUM FORTUITUM*

Material* (IP)	Interval between pretreatment and challenge†	Deaths at indicated numbers of days after infection							
Pyrexal 80 µg	24 Hours	2‡	—	—	—	—	—	—	—
Pyrexal 20 µg		5	23	28	28	—	—	—	—
Water		5	5	6	16	16	23	—	—
Pyrexal 80 µg	72 Hours	—	—	—	—	—	—	—	—
Pyrexal 20 µg		16	—	—	—	—	—	—	—
Water		6	21	22	25	30	35	—	—
Pyrexal 80 µg	1 Week	20	22	27	—	—	—	—	—
Pyrexal 20 µg		3	25	—	—	—	—	—	—
Water		3	3	3	3	4	4	4	5
Pyrexal 80 µg	4 Weeks	18	22	29	—	—	—	—	—
Pyrexal 20 µg		19	—	—	—	—	—	—	—
Water		5	13	13	13	18	21	—	—

* Injected I.P. in a final volume of 0.2 ml.
† Six day-old Tween albumin culture (0.05 ml) *Mycobacterium fortuitum* injected I.V. in final volume of 0.2 ml.
‡ Eight animals in each group. The sign — indicates that the animal was still living when the experiment was discontinued 4 weeks after infection.

ties of the circulating blood cells.[30] These data further suggest a close relationship between the effectiveness of thorium dioxide and the immunological state of the host.[31,3] It is true that von Jancso claimed in 1932 to have found in electrolytically dispersed copper a material that was able to destroy Kupffer's cells selectively and permanently,[15] but subsequent investigations have thrown some doubt upon the validity of his conclusions.[27,33]

A new avenue of experimental approach was opened by the discovery that endotoxins derived from Gram-negative bacteria are capable of protecting the experimental animal against a subsequent infection with unrelated Gram-positive agents such as the staphylococci, Gram-negative such as *Klebsiella pneumoniae* and atypical acid-fast bacilli such as *Mycobacterium fortuitum*.[34,35] Pursuing this line of investigation we found that the bacterial lipopolysaccharides as well as other bacterial substances of equal protective activity such

as pertu is vaccine and killed BCC stimulated the granulopectic activity of the RES[38,37]

The data that we obtained u ing a lipopolysaccharide derived from *Salmonella abortus equi* by treatment with concentrated phenol might erve as an illu trative example[38,40] This sub tance had been kindly supplied by Otto

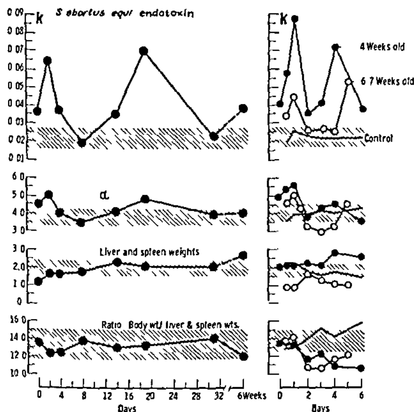


FIGURE 1 Comparative effect of treatment with Pyre al of infection with *Myc bac t m fort itum* on clea ance rate of carbon particles Lach point corr ponds to the a ith met c means of 5 animals the hatched l es nd cate the standard deviation fo the untreated c nt ols *Left* Pyrexal a lministered int aperitoneally in a final olume of 0 4 ml d stilled water *ight* Infective dose 0 05 ml of a 6-day-old Tween albumin culture of *M fo t tum* stra n Penso injected intravenously in a final volume of 0 2 ml distilled water

Westphal of the Dr A Wander Forschung institut Freiburg Sackingen Germany TABLE 1 demonstrates that this substance (Pyrexal) when given intraperitoneally prior to challenge in amounts as low as 20 μg protected male Swi s Rockefeller mice within 24 hours against an intravenous infection with *Mycobacterium fortuitum* This protective effe t lasted for at lea t 4 weeks

The data recorded on the left side of FIGURE 1 show that injection of this endotoxin into normal males of the Rockefeller Swiss train greatly stimulated

the clearance of colloidal carbon from the circulation as measured with the carbon pickup test of Halpern *et al.*[16] In brief, this test consists of the intravenous injection of 16 mg. colloidal carbon (India ink) of known particle size per 100 gm. body weight. In plotting the decrease of the logarithm of concentration against time one arrives at the value k which indicates the global clearance capacity of the RES, that is chiefly the liver and spleen. This value k stands in a third-order relationship to the quotient body weight/liver +

$$\alpha = \sqrt[3]{k} \times \frac{\text{body weight}}{\text{liver} + \text{spleen weights}}$$

spleen weights and the equation describes the clearance capacity of the RES of a given animal in relation to its body weight.

The right side of FIGURE 1 shows that infection produced a faster and more vigorous stimulation that was more pronounced in younger animals. The combined effects of endotoxin and subsequent infection resulted in a most striking stimulation, as might be expected. This effect was not additive. FIGURE 2 illustrates that in endotoxin pretreated mice, both global clearance k and the corrected phagocytic factor alpha rose without delay following challenge. Under the influence of the infection the weights of liver and spleen increased, while the body weights dropped. It will be recognized that the results were similar whether the interval between preinjection of endotoxin and challenge was 24 hours, 3 days, or 1 week.

The stimulus produced by a single injection of endotoxin had usually subsided after 4 weeks, that is, at a time when the experimental animal was still fully protected against challenge with *M. fortuitum*; this may be seen in FIGURE 3. Although the granulopectic activity of the RES had returned to control levels after 4 weeks, the protected animals responded with an immediate and conspicuous rise of their carbon clearance to the challenging infection. This immediate response effect was still demonstrable 8 weeks after the administration of Pyrexal. This can be recognized in FIGURE 3, where on the left side the results for a time interval of 4 weeks and on the right side those for a time interval of 8 weeks are plotted. With the same experimental system it was possible to show that similar results are obtained after the administration of pertussis vaccine, of whole killed BCG, and of subfractions of BCG.[36,37]

These data suggest a correlation between the ability to resist an infection and the ability to respond to it with a rise in granulopectic activity, since pretreatment with endotoxin undoubtedly affects both. It is well known, however, that endotoxins possess a profound influence on body temperature, blood picture, chemotaxis, coagulability, and the hypophyseoadrenal axis, all of which might very well exert an equally important effect upon nonspecific resistance. The complexity of these effects makes it difficult to decide what place should be assigned to the RES among the defense mechanisms that oppose the bacterial invader. An important contribution to this problem was made by Thorbecke, who found that global carbon clearance in germfree animals was not zero, as one might expect, but approximately one half that of the normal animal raised under natural conditions.[41,42] This finding suggests the

conclusion that the RES maintains a certain degree of granulopectic activity even in the absence of any bacterial stimulation

Investigation of genetically resistant or susceptible animals would be expected to shed further light on this problem since the former group should

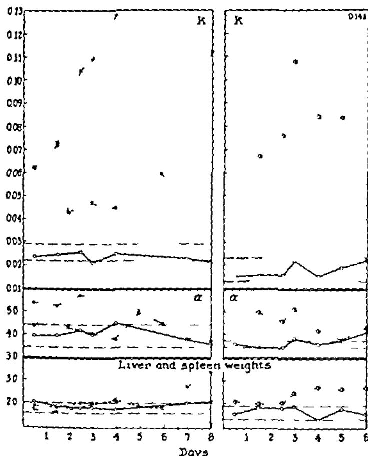


FIGURE 2 Short term effect of infection with *M fortuitum* on clearance rate of carbon particles in mice pretreated with Pyrexal Each point corresponds to the arithmetic means of 5 animals the broken lines indicate the standard deviation of the untreated controls *Left* the first group of animals received 20 μg of Pyrexal intraperitoneally in a final volume of 0 4 ml of distilled water and was challenged 12 hours later The second group received an identical dose of Pyrexal but was challenged 3 days later *Right* treated intraperitoneally with 20 μg Pyrexal in a final volume of 0 4 ml distilled water 1 week prior to challenge All 3 groups challenged with 0 05 ml of a 6-day old Tween albumin culture of *M fortuitum* strain Penso in a final volume of 0 2 ml distilled water

possess an RES of higher activity than the latter if the RES plays any role in nonspecific resistance

I examined this question in collaboration with Howard Schneider and Johanna M Lee of the Rockefeller Institute New York N Y In this investigation we employed the mouse strains BRVR and BSVS which are genetically resistant and susceptible to intraperitoneally injected *Salmonella typhimurium*[13]

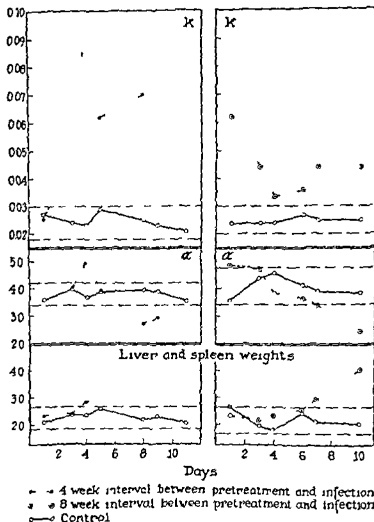


FIGURE 3 Long term effect of infection with *M* [illegible] clearance rate of carbon particles in mice pretreated with Pyrexal Th[illegible] the standard deviation of the untreated controls *Left* animals t[illegible]ated intrap[illegible] with 20 µg of Pyrexal in a final volume of 0.4 ml of distilled water and [illegible] *Right* mice pretreated intraperitoneally with 20 µg Pyrexal [illegible] 0.4 ml distilled water and challenged 8 weeks later Infecting dose for [illegible] 0.0[illegible] ml of a 6 day [illegible] Tween albumin culture of *M fortuitum* strain Penso su[illegible] final volume of 0.2 ml distilled water

To achieve a significant difference in survival in this model it is necessary to employ a double-strain inoculation. This consists of the administration of avirulent (strain RIA) followed 2 days later by virulent (strain SR 11) *S typhimurium*[41]. FIGURE 4 shows that all susceptible BSVS mice succumbed to the double strain inoculation by the twelfth day after administration of the avirulent RIA strain and by the tenth day after challenge with the virulent SR 11 strain. Thirteen of 23 resistant BRVR mice were still alive and in

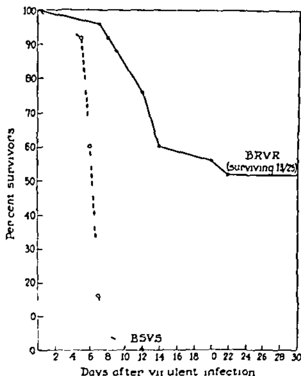


FIGURE 4 Survivorship after intraperitoneal infection with avirulent followed by virulent *S typhimurium*. Infected intraperitoneally with 1000 viable cells of avirulent strain RIA followed 2 days later by 100 000 cells of virulent strain SR 11. Reproduced from Böhme *et al*[41] by permission of the *Journal of Experimental Medicine*.

good condition at the time of the termination of the experiment that is 30 days after infection with the avirulent and 28 days after infection with the virulent strain

When the genetically resistant and susceptible mice were tested with respect to their carbon clearance a rather unexpected result was obtained. The left side of FIGURE 5 shows the carbon clearance curves for the susceptible BSVS animals. Their global clearance value *k* had risen to statistically significant level 1 day after the avirulent infection and responded with a further rise to the administration of the virulent strain on the second day. On day 4

however, the maximum response was reached and k dropped rapidly to control values on the sixth day A second rise was noted on the eighth day, when the last surviving animals were tested This point is difficult to interpret since it shows a high individual standard deviation The corrected phagocytic index α followed the same course as k The conspicuous drop in the ratio of body weight to liver plus spleen weights was caused chiefly by an increase in spleen weight

In contrast to this the resistant BRVR mice did not exhibit any rise in their granulopectic activity before the eighth day as illustrated on the right side of FIGURE 5 The global clearance value k reached a maximum around the fifteenth day and returned thereafter gradually to control values The

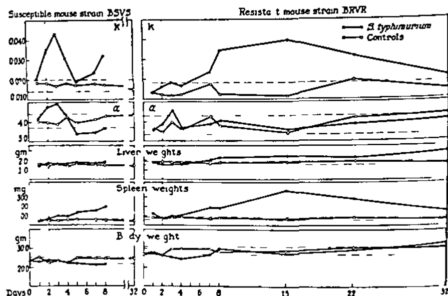


FIGURE 5 Time course studies in infected susceptible (BSVS) and resistant (BRVR) mice of global (k) and corrected carbon clearance rate (α) Broken lines indicate the standard deviation of the uninfected control

corrected phagocytic index α behaved much the same way As in the case of the susceptible BSVS mice a conspicuous increase in the spleen weights was observed that reached a peak about the fifteenth day but had returned to normal when the experiment was terminated

Macroscopic and microscopic inspection disclosed inflammatory lesions of equal character in the livers and spleens of both mouse strains These consisted predominantly of lymphocytes and monocytes and contained only few leukocytes Generally the hepatic changes were of comparable severity in both mouse strains but the splenic lesions were more numerous and more destructive in the susceptible BSVS animals Anatomically the resistant BRVR animals had overcome their disease by the end of the experiment Inflammatory lesions had all but disappeared and the only conspicuous vestige that remained was the presence of numerous thrombi in both liver and spleen

The results of these experiments stand in contrast to those obtained in the endotoxin pretreated and subsequently infected mice. Contrary to expectations the susceptible animals responded with a rapid and conspicuous rise of their granulopectic activity while the scavenger apparatus of the resistant animals was not brought into action until a time when all susceptible mice had already succumbed to their infection. Four interpretations appear to be possible.

First the carbon clearance test did not reflect the true state of activity of the RES. Second activity of the RES although truly reflected by the carbon clearance test is not necessarily connected with the ability to resist an infection with *S typhimurium*. Third for unexplained reasons bacterial multiplication and disintegration progressed much faster in the susceptible than in the resistant animal thus evoking an earlier stimulation of the RES. Finally the reticuloendothelial systems of the 2 strains are fundamentally different.

Benacerraf and his co workers have recently shown that heat killed suspensions of *Escherichia coli* and staphylococci labeled with I^{131} are cleared from the circulation in the same manner as colloidal carbon particles. This would suggest that the carbon pickup test gives a true picture of the state of activity of the RES[46]. The second objection cannot be answered definitely with the few data at hand but it seems to offer the most likely explanation. There is nothing to support the third argument, in fact the available evidence seems to indicate the contrary. *S typhimurium* multiplies in the spleens of susceptible and resistant animal equally well up to a certain point at which multiplication ceases in the resistant animals but continues in the susceptible ones[47]. Finally nothing indicates that the RES of the 2 mouse strains are fundamentally different. The initial granulopectic activity was identical in both mouse strains and the ability of both to form agglutinins against various Gram negative bacteria was found to be similar[48].

In summary it may be stated that preinjection of *Salmonella* endotoxin protects mice against subsequent infection with *Mycobacterium fortuitum*. At the same time the RES is conspicuously stimulated. The endotoxin seems to endow the reticuloendothelial cell in liver and spleen with an increased ability to respond to challenge with a rise in granulopectic activity for at least 6 weeks.

Experiments with mice genetically susceptible and resistant to *Salmonella typhimurium* showed on the other hand that the RES of the susceptible animals responded with considerably greater rapidity to the stimulus of infection than that of genetically resistant mice.

References

1 Wyssokowitsch W 1886 Über die Schicksale der ins Blut injizierten Mikroorganismen Z Hyg 1 3–45

1a Kiyono K 1914 Über vitale Karminspeicherung 258 pp Fisher Jena Germany

2 Maximow A 1924 Tuberculosis of mammalian tissue *in vitro* J Infectious Diseases 34 549–584

3 Doan C A & F R Sabin 1927 Local progression with spontaneous regression of tuberculosis in the bone marrow of rabbits correlated with a transitory anemia and leucopenia after intravenous inoculation J Exptl Med 46 315 341

4 Lurie M B 1942 The fate of tubercle bacilli ingested by mononuclear phagocytes derived from normal and immunized animals J Exptl Med 75 247 267

5 Mackaness G B 1952 The action of drugs on intracellular tubercle bacilli J Pathol Bacteriol 64 429–446

6 FURNESS G & I FERREIRA 1959 The role of macrophages in natural immunity to Salmonellae J Infectious Diseases 104 203–206
7 SABIN F R C A DOAN & C F FORKNER 1930 Studies on tuberculosis J Exptl Med Suppl 3 Chap IV
8 SUTER E 1953 Multiplication of tubercle bacilli within mononuclear phagocytes in tissue cultures derived from normal animals and animals vaccinated with BCG J Exptl Med 97 235 245
9 FONG J P SCHNEIDER & S S ELBERG 1956 Studies on tubercle bacillus monocyte relationship I Quantitative analysis of serum of animals vaccinated with BCG upon bacterium monocyte system J Exptl Med 104 455–465
10 GELZER J & E SUTER 1959 The effect of antibody on intracellular parasitism of *S typhimurium* in mononuclear phagocytes *in vitro* J Exptl Med 110 715 730
11 WOLF HEIDEGGER G 1941 Zur Form und Lagerung der Kupfferschen Sternzellen Z mikroskop anat Forsch 50 623–635
12 WACHTER H P 1956–1957 Frühe Reaktionen der Leber nach intravenoser Tuschein jektion und anderen Reizen Jahrb Borstel 1956/57 123–155
13 HAMAZAKI Y & M WATANABE 1929–1930 Über die Affinität der Histiozyten fur verschiedene Organe und Gewebe Folia Haematol 39 12–31
14 DE HAAN J & I A HOEKSTRA 1928 Versuche über die Herkunft und die Deutung der Kupfferschen Sternzellen der Leber Arch exptl Zellforsch Gewebezucht 5 35–45
15 VON JANCSÓ N 1955 Speicherung Stoffanreicherung im retikuloendothelialen System und der Niere 34–39 Akademiai Kiádó Budapest
16 HALPERN B N G BIOZZI G MENE & B BENACERRAF 1951 Étude quantitative de l activité granulopexique du systeme SRE par l injection intraveineuse d encre de Chine de particules de carbone de dimensions connues Ann inst Pasteur 80 582 604
17 BIOZZI G B N HALPERN C STIFFEL & D MOUTON 1958 Quantitative study of the metabolic activity of the Kupffer cells on a heat denatured serum albumin labelled with I^{131} Brit J Exptl Pathol 39 510–518
18 STIFFEL C 1958 Etude de la fonction phagocytaire du systeme reticuloendothelial J Physiol 50 911–949
19 WACHSTEIN M & E MEISEL 1959 Enzymatic histochemistry of ethionine induced liver cirrhosis and hepatoma J Histochem 7 189–201
20 ROUS P & J W BEARD 1934 Selection with the magnet and cultivation of reticulo endothelial cells (Kupffer cells) J Exptl Med 59 577–591
21 BEARD J W & P ROUS 1934 The character of Kupffer cells living *in vitro* J Exptl Med 59 593–607
22 KRITSCHEWSKI I L & S I SHAPIRO 1939 Über die Natur der Immunitat bei Rückfallfieber XI Über die Schutzfunktion der nicht mit Eisenzucker blockierten Histiozyten des ausserhalb der Milz gelegenen Teiles des RES Z Immunitatsforsch 65 264–275
23 KRITSCHEWSKI I L & I L RUBINSTEIN 1931 Über die Natur der Immunitat bei Rückfallfieber XV Über die Bedeutung des Retikuloendothelial systems als einer Abwehrvorrichtung wahrend der erworbener Immunitat bei Ruckfallfieber Z Immunitatsforsch 71 372–382
24 KRITSCHEWSKI I L & J S MEERSON 1937 Über die Bedeutung des retikuloendothelialen Apparates bei Infektionskrankheiten IX Die Rolle des Retikuloendothelial systems bei Vogelmalaria Z Immunitatsforsch 76 499 50
25 RUSSEFF C 1934 Das retikuloendotheliale System und die natürliche Immunitat der weissen Mause bezüglich des Bazillus [illegible] anthracis Z Immunitätsforsch 82 141 145
26 WEILAND P 1936 Welche Bedeutung [illegible] retikuloendothelialen System bei der aktiven Immunisierung gegen [illegible] zum [illegible] Z Immunitatsforsch 88 460–471
27 KRITSCHEWSKI I L R S TSCHERIKOWER & K M DAWIDOWSKAJA BARISCHEWA 1933 1934 Über eine noch unbekannte Funktion des Retikuloendothelialsystems XIX Z Immunitätsforsch 81 163 184
28 FINE J S RUTENBURG & F B SCHWEINBURG 1959 The role of the reticuloendothelial system in hemorrhagic shock J Exptl Med 110 [illegible]
29 ZWEIFACH B W B BENACERRAF & L THOMAS 19[illegible] The relationship between the vascular manifestations of shock produced by endotoxin trauma and hemorrhage II The possible role of the reticuloendothelial system in each type of shock J Exptl Med 106 403–414
30 HELD A 1933 Die Funktionshemmung des RES Z ges exptl Med 88 [illegible]

31 HANKE H 1932 Experimentelle Untersuchungen biologischer Abwehrvorgänge bei Thoriumdioxydspeicherung des Retikuloendothels I Das Verhalten normergischer Tiere Z ges exptl Med 85 623–652
32 HANKE H 1932 Experimentelle Untersuchungen biologischer Abwehrvorgänge bei Thoriumdioxydspeicherung des Retikuloendothels II Das Verhalten hyperergischer Tiere Z ges exptl Med 85 653–673
33 LETTERER F 1933 Über die Wirkung von elektrokolloidalem Kupfer auf das retikuloendotheliale System Klin Wochenschr 12 59 –600
34 DUBOS R J & R W SCHAEDLER 1956 Reversible changes in the susceptibility of mice to bacterial infections J Exptl Med 104 53–65
35 BOHME D & R J DUBOS 1958 The effect of bacterial constituents on the resistance of mice to heterologous infections and on the activity of their reticuloendothelial system J Exptl Med 107 532 536
36 BOHME D & C A BOUVIER Über den Einfluss bakterieller Fraktionen auf Gewebe retikuloendotheliales System und Differentialblutbild der Albinomaus Beitr Allg pathol u Pathol Anat In press
37 BOHME D & C A BOUVIER The influence of killed Myco tuberculosis (BCG) and its constituents upon the reticuloendothelial system internal organs and percentage distribution of leucocytes in normal albino mice Proc 3rd Intern Symposium for the Reticuloendothelial System Ronald New York N Y In press
38 WESTPHAL, O O LUDERITZ & F BISTER 19 2 Über die Extraktion von Bakterien mit Phenol Wasser Z Naturforsch 7B 148 156
39 WESTPHAL, O & O LUDERITZ 1954 Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien Angew Chem 66 407–417
40 FROMME I O LUDERITZ A NOWOTNY & O WESTPHAL 1958 Chemische Analyse des Lipopolysaccharids aus Salmonella abortus equi Pharm Acta Helv 33 391–400
41 THORBECKE G J 19 9 Some histological and functional aspects of lymphoid tissue in germfree animals I Morphological studies Ann N Y Acad Sci 78(1) 23 246
42 THORBECKE G J & B BENACERRAF 19 9 Some histological and functional aspects of lymphoid tissue in germfree animals II Studies on phagocytosis *in vivo* Ann N Y Acad Sci 78(1) 247–253
43 WEBSTER L T 1937 Inheritance of resistance of mice to enteric bacterial and neurotropic virus infection J Exptl Med 65 261 28
44 SCHNEIDER H 1949 Nutrition of the host and natural resistance to infection IV The capability of the double strain inoculation test to reveal genetically determined differences in natural resistance to infection J Exptl Med 89 52 539
45 BOHME D H SCHNEIDER & J M LEE 1959 Some physiopathological parameters of natural resistance to infection in murine salmonellosis J Exptl Med 110 9 26
46 BENACERRAF B M S SEBESTYEN & S SCHLOSSMANN 1959 A quantitative study of the kinetics of blood clearance of P[32] labelled *E coli* and staphylococci by the RES J Exptl Med 110 27–48
47 SCHNEIDER H & N D ZINDER 1956 Nutrition of the host and natural resistance to infection V An improved assay employing genetic markers in the double strain inoculation test J Exptl Med 103 207 223
48 HUDACK S S & P D MCMASTER 1934 Agglutinin formation within the lymph nodes of resistant and susceptible mice Proc Soc Exptl Biol Med 31 753 755

BACTERIAL PHAGOCYTOSIS BY THE RETICULOENDOTHELIAL SYSTEM *IN VIVO* UNDER DIFFERENT IMMUNE CONDITIONS*

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In the past few years much has been learned about the phagocytic activity of the cells of the reticuloendothelial system (RES) in contact with the blood from experiments dealing with the kinetics of blood clearance of standardized suspension of carbon by various organs of the RES[1,2] When such avidly phagocytized colloidal suspensions of homogeneous particle size are injected intravenously about 90 per cent of the material is removed by phagocytes of the liver and spleen and the rate of clearance follows an exponential function of the time $K = (\log C_1 - \log C_2)/T$ If small numbers of these particles are injected the rate of clearance is very rapid, and the Kupffer s cells of the liver, because of the large blood supply of that organ remove nearly all the injected material with a maximum efficiency in a single passage through the sinusoids of the order of 80 to 90 per cent depending upon the animal species[3] In this range of dosage the colloid clearance test provides a good technique to measure liver blood flow[4,5] As larger amounts are injected to challenge all the phagocytic cells with an excess of particles a critical dose is reached above which the rate of blood clearance K becomes dose-dependent ($K \times D =$ constant) as the result of the combined effects of the decreasing efficiency of the phagocytic cells to clear the blood of larger concentrations of colloids and of the saturating effect of phagocytized particles on the RES[2] These effects acting in opposite directions cancel themselves out and the clearance continues to follow an exponential function of the time In this range of dosage above the critical concentration the colloid clearance technique may be used to measure the phagocytic capacity of the RES This test was the basis for many studies of the phagocytic activity of the RES in experimental infections[6,7] Several infectious processes are associated with a considerable increase in the phagocytic activity of the RES at least in an early phase later in the case of virulent organisms or high infective dose the phagocytic function is depressed Other experiments have also shown that the rate of clearance of carbon by the RES is greatly increased after treatment with agents that improve natural resistance to infections such as bacterial lipopolysaccharides[8,10] a polysaccharide extract of yeast cell wall (zymosan)[11] or BCG infection[11,1]

The fate of bacteria injected intravenously and the mechanism of their disappearance from the circulation have been studied in various animal species by many investigators Their observations have shown that (1) bacteria can be cleared from the blood very rapidly and in very large numbers by the RES of the liver and spleen[13,1] (2) previous immunization of the animals increases greatly the ability of the RES to extract both avirulent and virulent bacteria from the blood and improves its ability to kill the phagocytized organisms[13,18,21]

The work reported in this paper was supported in part by Grant E 2094 from the National Institute of Allergy and Infectious Diseases Public Health Service Bethe[illegible] d

and (3) the rapid clearance of injected bacteria is followed in the case of virulent organisms by a second phase when the blood concentration of bacteria increases in spite of the host's defenses probably as a result of bacterial multiplication within the phagocytes.[14-1]

Only the events occurring in the first phase of rapid bacterial clearance and the factors that influence them have been investigated in the experiments reviewed here.

The techniques and approaches used in the investigation of phagocytosis of colloidal particles have been applied to a quantitative study of bacteria[22] and virus[23] clearance from the blood by the RES using P^{32} labeled microorganisms in an attempt to investigate the various factors that influence these processes. From the experience with colloidal particles the kinetics of clearance of bacteria or viruses by the RES would be expected to depend upon the following three factors: (1) the homogeneity of the suspension, (2) the efficiency with which the microorganisms could be phagocytized (determined in large part by the effect of serum opsonic factors), and (3) the dose injected.

Blood Clearance of Bacteria by the RES

A study was performed of the blood clearance of P^{32} labeled *Staphylococcus aureus* in mice and of *Escherichia coli* in mice, guinea pigs, and rabbits. Heat killed staphylococci and live or heat killed *E. coli* were used. No significant difference was observed in mice between the results obtained with live and heat killed *E. coli*.

When P^{32} labeled *E. coli* or staphylococci are injected intravenously, the blood radioactivity measuring the number of bacteria decreases according to an exponential function of the time down to a concentration of about 10 per cent. The curve in FIGURE 1 represents the result of typical experiments in mice. Most of the bacteria are cleared from the blood as a homogenous suspension. As for the clearance of colloidal particles the equation given above can be used to describe bacterial clearances within the limits of the experimental conditions defined above in which K, referred to as the phagocytic index, measures the rate of clearance of the substrate used. The results shown in FIGURE 1 further show that while *E. coli* disappear very slowly from the blood of normal mice, a comparable number of staphylococci are cleared very rapidly by the RES. The reason for this basic difference is that Gram negative organisms such as *E. coli* are phagocytized very poorly unless they are adequately opsonized by serum factors such as antibody and complement as shown in the experiments described below. The level of antibodies against *E. coli* is very low in mouse serum (agglutination titer 1:4). Phagocytosis of staphylococci by the RES may not require previous opsonization as shown by Howard and Wardlaw.[4,5] These authors observed that staphylococci could be efficiently phagocytized by the Kupffer cells of rat livers perfused with the bacterial suspension in Ringer's solution in the absence of serum, while removal of *E. coli* by the same liver preparation required both heat-stable and heat labile components of serum.[4,5]

P^{32} labeled *E. coli* or staphylococci injected intravenously in mice are phagocytized for the most part by the reticuloendothelial cells of the liver and spleen (FIGURE 2) and a small amount (about 5 per cent) is found in the lungs and

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traces in the kidneys However the respective amounts phagocytized by the liver or the spleen for a given dose vary with the rate of clearance of the bacteria that is with the efficiency with which they can be extracted in one passage by the principal filtering organs that is the liver and the spleen Well

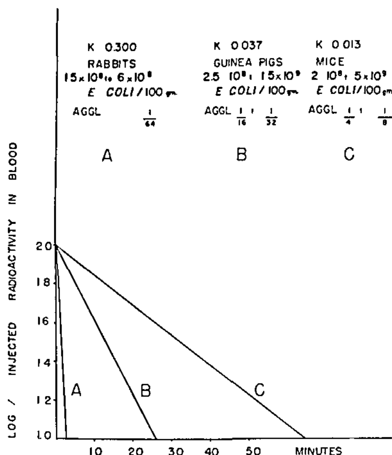


FIGURE 3 Average blood clearances of I[131] labeled *E coli* in mice guinea pigs and rabbits Key A rabbits 1 5 to 6 $\times$ 10[8] *E coli*/100 gm K = 0 30 B guinea pigs 2 5 $\times$ 10[8] to 1 5 $\times$ 10[9]/100 gm K = 0 037 C mice 2 $\times$ 10[8] to 5 $\times$ 10 /100 gm K = 0 013
Reproduced from Benacerraf *et al* [22] by permission of the *Journal of Experimental Medicine*

opsonized or otherwise easily phagocytized bacteria can be removed with great efficiency by the liver therefore because of its very large circulation this organ becomes the principal filter for such bacteria In the case of bacteria that are not avidly phagocytized such as poorly opsonized *E coli* in normal mice the share removed by the spleen phagocytes increases considerably at the expense of the liver The spleen because of its slower circulation and better chances of contact between bacteria and macrophages is a more efficient filter for bac

LOG COUNTS/MIN IN 0 05 BLOOD

3 4
3 2
3 0
2 8
2 6
2 4
2 2
2 0
1 8
1 6

E COLI 10⁹/100 gm
K 0 016

STAPHYLOCOCCI
K 0 14

10 20 30 40 50 60 70 80 MINUTES

FIGURE 1 Blood clearances in 2 normal mice of 10^9 heat killed *E coli* per 100 gm and of a dose of heat killed staphylococci equivalent to 4×10^8 *E coli* per 100 gm with respect to optic density measurement
Reproduced from Benacerraf *et al* [22] by permission of the *Journal of Experimental Medicine*

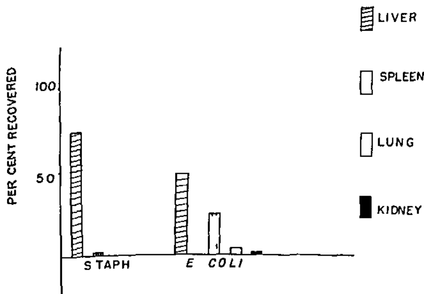


FIGURE 2 Percentage of the injected radioactivity recovered in the organs of normal mice injected with 10^9 P^{32} labeled *E coli*/100 gm or with an amount of I^{131} labeled staphylococci equivalent to 4×10^8 *E coli*/100 gm with respect to optical density measurement
Reproduced from Benacerraf *et al* [22] by permission of the *Journal of Experimental Medicine*

LOG COUNTS / MIN IN 0.05 ml BLOOD

$\frac{K_1}{K_2}$ $\frac{0.011}{0.004}$ 2.7

3.0
2.8
2.6

10 20 30 40 50 60 MINUTES

E. COLI 10^9/100 gm

↓ CARBON 16 mg/100 gm

$\frac{K_1}{K_2}$ $\frac{0.014}{0.007}$ 2

3.0
2.8
2.6

10 20 30 40 50 60 MINUTES

FIGURE 4 Competition exerted by the injection of carbon particles (16 mg./100 gm body weight) on the phagocytosis of I[32] labeled *E. coli* by the RES in mice
Reproduced from Benacerraf *et al* [32] by permission of the *Journal of Experimental Medicine*

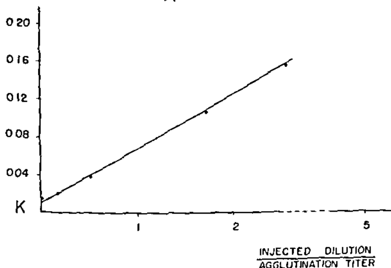


FIGURE 5 Effect of mouse antibody against *E. coli* lipopolysaccharide injected intravenously in mice (0.5 ml.) on the rate of clearance of *E. coli* from the blood by the RES measured by the phagocytic index K for the dose of 10 bacteria/100 gm
Reproduced from Benacerraf *et al* [32] by permission of the *Journal of Experimental Medicine*

teria that are less avidly phagocytized. If *E. coli* are opsonized with an adequate amount of antibody the rate of clearance is rapid and, as in the case of staphylococci, the liver contains nearly all the injected radioactivity. It is interesting to note that this difference in behavior between the liver and spleen has been observed already in the study of phagocytosis of colloidal particles.[21,26] It is found generally that unstable, avidly phagocytized colloids have almost exclusively a liver clearance if injected in small amounts, while well stabilized colloids are removed in greater amounts by the spleen and bone marrow.

If the rates of clearance of similar numbers of *E. coli* are compared in mice, guinea pigs, and rabbits and related to the level of antibodies in the sera of these animals (FIGURE 3), the following conclusions may be drawn: the rate of clearance of *E. coli* in mice is slow, without considerable variations in a range of dosage from 2×10^8/100 gm. to 5×10^9/100 gm., $K = 0.013$. Contrary to this, the rates of clearance of the same bacteria, 1.5×10^8 to 6×10^9/100 gm., are very rapid in rabbits, where K is greater than 0.300. Correspondingly, the agglutination titers against *E. coli* are 1/4 to 1/8 in normal mouse sera and 1/32 and 1/64 in normal rabbit sera. Guinea pigs show a rate of clearance of *E. coli* that is intermediate between what is observed in mice and rabbits, and the agglutination titers of guinea pig sera range between 1/16 and 1/32. It seems therefore that the most important factor to consider in the clearance of *E. coli* is the level of antibodies, which becomes rate limiting unless present in adequate amount to opsonize the bacteria properly.

It appears that in the range of dosage investigated the rate of clearance is independent of the number of bacteria injected. The capacity of the RES for clearing bacteria from the blood is of considerable magnitude. However, the rate-decreasing effect of large numbers of bacteria can be demonstrated, as in the case of colloidal particles, for very large doses (5×10^9 *E. coli*/100 gm.) in mice and rabbits, provided a sufficient amount of antibody is present so that it is no longer the limiting factor.[illegible]

To verify whether bacteria and colloidal particles such as carbon are phagocytized by the same mechanism and by the same cells of the RES, the competitive effect of carbon particles on the phagocytosis of *E. coli* was investigated. This phenomenon of competition had been observed when 2 colloids, such as carbon or iron oxide particles, that are phagocytized by the RES with comparable avidity, are both present in the circulation in suitable amounts.[8] The results presented in FIGURE 4 illustrate that, as soon as 16 mg. carbon is injected, the rate of clearance of *E. coli* (10^9 bacteria/100 gm.) decreases; the ratio (K_1/K_2) of the values of K before and after the injection of the carbon suspension measures the degree of interference exerted by the carbon particles on the phagocytosis of *E. coli*.

Effect of Antibody and Complement on the Blood Clearance of E. coli by the RES

The data presented above have shown that the limiting factor that determines the rate of phagocytosis of *E. coli* by the RES is the level of antibody in the serum. To investigate further the opsonizing effect of antibody, use has been made of the fact that mice possess naturally very low levels of antibodies against *E. coli*, and that the blood clearance of these bacteria is therefore very slow in this animal species.

titer of 1/1800 and the chicken antiserum in a titer of 1/6400. The results of these experiments are presented in FIGURES 6, 7 and 8. Both chicken and rabbit antibodies increase the rates of clearance of *E. coli* from the blood of mice. This effect is proportional to the amount of antibody injected. Chicken anti-*E. coli* is, however, far less efficient than rabbit antibody, as it seems to require about 10 times more antibody in terms of agglutinating units than rabbit antibody to bring about the same degree of opsonization (FIGURE 8). A close study of the bacterial clearance curves themselves reveals that when the proper dose of antibody is used, a latent period is observed before the opsonizing effect of the antibody increases sharply the clearance rate. This time lag is only 4 min. with rabbit antibody, but may be as long as 15 min. in the case of chicken antibody. As shown below, this is the time required for complement to exert its opsonizing effect. The difference in efficiency between the opsonizing properties of rabbit and chicken antibody appears to be therefore related to their relative capacity to bind mouse complement.

In order to analyze further the reason for the lag period, experiments were performed where the bacteria were incubated *in vitro* with an adequate dilution of chicken antibody (1/2000) in the presence of normal mouse serum or of mouse serum inactivated either by heating at 56° C. for 30 min. or by treatment with zymosan at 37° C. to bind C'3. These experiments were carried out with live P^{32} labeled *E. coli*, as it was found that prolonged incubation of heat killed bacteria brought about some loss of the P^{32} label from the bacteria. When live *E. coli* were used, the loss of P^{32} from the bacteria was negligible.

The results of these experiments are presented in FIGURE 9 and TABLE 1. They show that when *E. coli* were incubated with chicken antibody and fresh mouse serum for 30 min. *in vitro*, the rate of clearance was rapid immediately after injection and no lag period was observed. If the bacteria were incubated with the same dilution of chicken antibody, but with heat inactivated or zymosan treated mouse serum before injection into mice, the same latent period of about 15 min. was observed as when chicken antibody was injected into mice previous to the injection of bacteria. The lag period is therefore due to the time necessary for the opsonizing action of the mouse complement system and illustrates the fact that complement is needed in addition to antibody to opsonize *E. coli* for phagocytosis by the RES. In these experiments it was observed also that much less antibody is required for adequate opsonization if the bacteria are incubated with antibody *in vitro* than if antibody is injected *in vivo*.

The Effect of Endotoxin on the Clearance of E. coli from the Blood and on the Level of Opsonizing Serum Antibody

To study the mechanism of the increase in natural resistance against bacterial infection observed after treatment with endotoxin, the clearance of *E. coli* from the blood was investigated in mice that had been treated with a bacterial endotoxin[22] and whose polysaccharide moiety was known not to cross react with *E. coli*. The results of these experiments are shown in TABLE 2. Following treatment with lipopolysaccharide, the rate of clearance of *E. coli* was very much increased. This effect could be transferred to normal mice by the serum of endotoxin treated mice and is therefore related to an increase in opsonic

Anti *E coli* sera were prepared in mice, rabbits, and chicken and the effect of their antibodies was assayed by injecting mice intravenously with 0 5 ml of various dilutions of these antisera before the standard dose of P^{32} labeled *E coli* (10^9/100 gm) used for clearance[10] It was found that mouse and rabbit antibody were about equally effective in enhancing phagocytosis of *E coli* by the RES The rates of clearance K were increased by the previous administration of antibody This effect varied linearly with the dose of antibody in

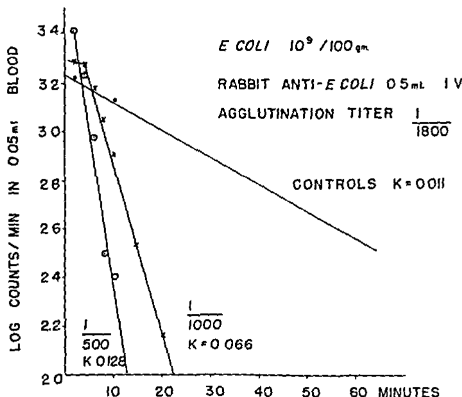


FIGURE 6 Effect of various dilutions of rabbit anti *E coli* serum injected intravenously in mice (0 5 ml) on the rate of clearance of P^{32} labeled *E coli* from the blood by the RES (agglutination titer 1/1800)

jected until a maximum rate clearance was reached that could not be improved upon by larger antibody concentrations (FIGURE 5) As little as 0 01 μg rabbit antibody nitrogen provided a sufficient level of antibody in the serum for optimal opsonization so that the bacteria were cleared at the maximum possible rate

In order to investigate the possible role of complement in the process of opsonization the effect of chicken anti *E coli* antibody which fixes mouse complement very poorly was compared with that of rabbit antibody which fixes complement adequately The rabbit serum used agglutinated *E coli* in a

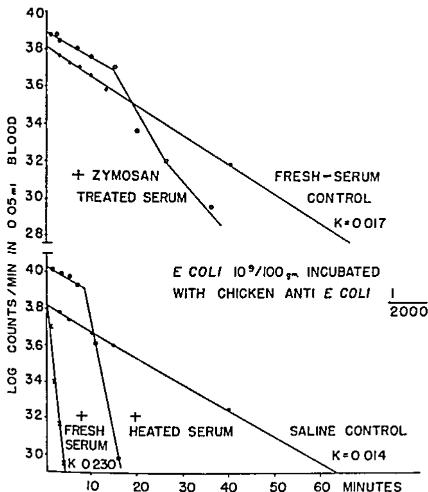


FIGURE 9 Effect of chicken anti *E coli* serum (agglutination titer 1/6400) and of fresh mouse serum as a source of complement on the *in vitro* opsonization of *E coli* for phagocytosis by the RES Control suspensions were incubated either in saline or fresh mouse serum without chicken antibody Note the absence of a lag period before rapid clearance occurs in the case of bacteria opsonized with chicken antibody and fresh mouse serum Lag period is observed if heated or zymosan treated mouse serum is used

TABLE 1
PER CENT RECOVERY OF RADIOACTIVITY AFTER INJECTION OF 10 *E coli*/100 GM INCUBATED IN VARIOUS MEDIA

	Saline control	Fresh serum control	Chicken anti *E coli* 1/2000 +		
			Fresh serum	Heated serum	Zymosan serum
Liver	45	43	68	52.5	27
Spleen	16.5	16.5	2	6.8	8
Lungs	2.5	2.5	2.5	3.6	5
Kidneys	1	1	1	0.5	6.4
Blood	13	9	5	5	10
Total	78	72	78.5	68.4	56

LOG COUNT/MIN IN 0 05 ml BLOOD

36 34 32 30 28 26 24 22 20

10 20 30 40 50 60 MINUTES

1/5000 K-0 014

1/1000 K 0 061

1/300 K 0 185

CHICKEN ANTI *E COLI*

E COLI 10^9 /100 gm

FIGURE 7 Effects of various dilutions of chicken anti *E coli* serum injected intravenously in mice (0 5 ml) on the rate of clearance of F^3 labeled *E coli* from the blood by the RES (agglutination titer 1/6400)

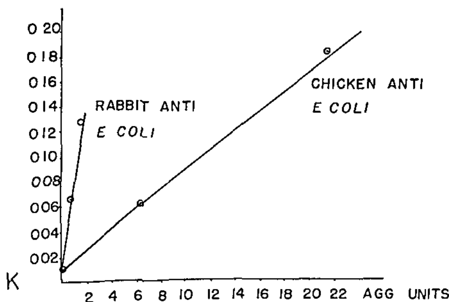


FIGURE 8 Comparison of the opsonic actions of rabbit and chicken anti *E coli* antibodies measured by their effects on the phagocytic index *K* for the dose of 10 *E coli* per 100 gm body weight

11 Biozzi C B Benacerraf F Grumbach B N Halpern J Levaditi & N Kit 1954 *Ann inst Pasteur* 87 291
12 Dubos R J & R W Schoedler 1957 J Exptl Med 106 703
13 Bull C C 1915 J Exptl Med 22 4 5
14 Wright H D 1927 J Pathol Bacteri l 30 185
15 Sullivan F I I F Nekerman & I R Cannon 1934 J Immun l 26 9
16 Kerby C I B C Holland & S I Martin 1950 J Immun l 61 123
1 Hollingworth J W & B I Beeson 1955 Yal J Biol and Med 28 56
18 Bennett I I & B I Beeson 1953 Yale J Biol and Med 26 41
19 Rogers D I & M A Melly 1957 J Exptl Med 105 113
20 Rogers F 1958 The Pasteur Fermentation Centennial (185 19 ... Pfizer & Co Brooklyn N Y
21 Teale F H 1935 J Immunol 28 133
22 Benacerraf B M M Sebestyen & S Schlossman 19 9 J Exptl ... 110 ?
23 Brunner T D Hurez R T McCluskey & B Benacerraf J I ... In press.
24 Howard J G & A C Wardlaw 19 8 Immunology 1 338
25 Wardlaw A C & J G Howard 19 9 Brit J Exptl Path 40 113
26 Dobson F L 1957 Physiopathology of the Reticulo-Endothelial System A Symposium 80 Thomas Springfield Ill
27 Biozzi C B Benacerraf B N Halpern & C Stiffel 19 7 R E S Bull 3 3
28 Rowley D 1959 J Exptl Med 11 137

factors in the serum, correlated with a rise in the agglutinating titer against *E. coli*. The increase in serum opsonins appears to be immunologically not specific as it was elicited by endotoxins without known cross reactivity with *E. coli*. Similar increases in the level of nonspecific opsonins in the sera of mice treated with endotoxin have been observed by Rowley [9] and by C. Jenkin (personal communication) using *Salmonella typhimurium*, *Vibrio comma* and intraperitoneal macrophages. The nonspecific nature of these opsonins suggests that unsuspected cross reactivity may exist between all endotoxins, and that it may be related to the lipid portion of the lipopolysaccharide molecule. While the increase in these opsonic factors in serum following endotoxin is well established, their role in nonspecific resistance should be evaluated.

TABLE 2

EFFECT OF ENDOTOXIN ON THE RATE OF CLEARANCE OF *E. COLI* FROM THE BLOOD AS MEASURED BY THE PHAGOCYTIC INDEX K FOR 10 BACTERIA/100 GM

Endotoxin treatment γ/mouse	Time after first endotoxin injection	No. of mice	Phagocytic index K Mean values	Agglutinin titer
58 Control mice			0.015 ± 0.005	1/4 to 1/8
S. typhosa				
25	3 Hours	3	0.0[illegible]	
	10 Hours	2	0.016	
	24 Hours	3	0.0[illegible]	
	6 Days	4	0.03[illegible]	
10 and 100 × 3	10 Days	1[illegible]	0.110	1/64
0.5 ml. serum transfer		4	0.057	
Serratia marcescens				
10 and 100 × 3	10 Days	4	0.130	
0.5 ml. serum transfer		2	0.093	
S. typhimurium				
10 and 100 × 2	1 Week	4	0.058	
Mouse polysaccharide				
10 and 100 × 3	10 Days	5	0.015	

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References

1. BIOZZI, G., B. BENACERRAF & B. N. HALPERN. 1953. Brit. J. Exptl. Pathol. 34: 441.
2. BENACERRAF, B., G. BIOZZI, B. N. HALPERN & C. STIFFEL. 1957. Physiopathology of the Reticulo Endothelial System. A Symposium: 52. Thomas. Springfield, Ill.
3. BENACERRAF, B., B. N. HALPERN, G. BIOZZI, C. STIFFEL & D. MOUTON. 1957. Brit. J. Exptl. Pathol. 38: 35.
4. BIOZZI, G., B. BENACERRAF, B. N. HALPERN, C. STIFFEL & B. HILLEMAND. 1958. J. Lab. Clin. Med. 51: 230.
5. HALPERN, B. N., G. BIOZZI, G. PEQUIGNOT, B. DELALOYE, C. STIFFEL & D. MOUTON. 1959. Pathol. Biol. 7: 163.
6. BIOZZI, G., B. N. HALPERN, B. BENACERRAF & C. STIFFEL. 1957. Physiopathology of the Reticulo-Endothelial System. A Symposium: 204. Thomas. Springfield, Ill.
7. BOHME, D. & P. J. DUBOS. 1958. J. Exptl. Med. 107: 523.
8. BIOZZI, G., B. BENACERRAF & B. N. HALPERN. 1955. Brit. J. Exptl. Pathol. 36: 226.
9. ROWLEY, D. 1955. Lancet 1: 232.
10. LANDY, M. 1956. Federation Proc. 15: 598.
11. BENACERRAF, B. & M. M. SEBESTYEN. 1957. Federation Proc. 16: 860.

begin 24 hours after injection and reached a peak (30 to 50 per cent) 48 hours after injection but was back to normal values by the fifth day There was no significant difference histologically in bone marrow and kidney in comparison with that of the control group

Lung

Twenty four hours cells constituting the alveolar septa of lung became enlarged and increased in number resulting in extending the width of the alveolar septa twofold to tenfold This extension was diffuse or occasionally nodular There was an occasional leukocytic accumulation in the alveolar septa Endothelial cells of arterioles frequently became rounded and increased in number Hyperplasia of cells in the perivascular space of arterioles also was so significant that occasionally they formed cellular nodules surrounding the vessels A few leukocytes migrated into these nodules but not many Alveoli and bronchial lumen remained virtually clear Vascular congestion of the lungs as a whole was not conspicuous

Forty eight hours cellular hyperplasia of alveolar septa became more extreme and more diffuse Mitotic figures of these proliferating cells were often encountered Increase in the number of endothelial cells and adventitial cells of arterioles also became more significant Consequently some arterioles were obliterated by these cells and sometimes one could see thick concentric arrangement of adventitial cells around the arterioles (FIGURE 1) Strange to say however in the lung of one case in which there was bronchopneumonitis there was not a conspicuous cellular response as presented by the others

Five days the number of cells of the alveolar septa of lung that had proliferated began to decrease consequently the alveolar septa of normal width appeared here and there Mitotic figures were obscure Although numerous cells in the intima and adventitial spaces of arterioles still remained these proliferated cells seemed to be more or less in the process of degenerating In one case where there was a bronchopneumonitis the above mentioned findings were not observed Instead there was infiltration of leukocytes limited to the bronchi and in the nearby lung tissue

Liver

Twenty four hours capillaries in the acini of liver showed slight congestion and a few granulocytes were present Kupffer s cells and the endothelial cells of the capillaries were somewhat swelled and the chromatin volume of their nuclei was increased to some degree Cell bodies of Kupffer s cells were short and spindle shaped ovoid or nearly round Occasionally Kupffer s cells with two nuclei appeared Cells of hepatic arterial walls also began to proliferate

Forty eight hours Kupffer s cells were significantly increased in number They were sometimes arranged syncytially and also in some small nodules that consisted of the proliferation of histiocytes These also occurred in interstitial tissue many of them showed a radial cell arrangement around the hepatic artery A few granulocytes were present also in capillaries

Five days hyperplasia of Kupffer s cells was more remarkable they not only increased in number diffusely but resulted in many different-sized nodules

MORPHOLOGIC CHANGES ACCOMPANYING RES STIMULATION*

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Introduction

Experiments in our laboratories have indicated that specially prepared cell wall fractions derived from yeast (restim) can stimulate the RES This stimulation applies not only to phagocytosis but also to antibody synthesis[1] (see also J H Heller this monograph) For some time chemical investigation aimed at isolating and identifying the active component in these cells has been under way Although these experiments are still in progress it can be stated that the active component appears to be a lipid fraction which is free from polysaccharide and protein

This lipid fraction has been shown to be able to stimulate the RES Hence it was of interest to examine morphologic concomitants of such stimulation in order to explore the obvious inference that the active lipid fraction represented the stimulatory component in restim

Methods and Materials

Thirty one male white, 150 gm CFN rats were intravenously injected with 3 mg of restim (J H Heller this monograph) suspended in 0 3 ml of saline Seven rats were autopsied at 24 hours, 48 hours and 5 days after injection Several organs (lung liver spleen kidney lymph nodes and bone marrow) were removed and histologically examined These organs were fixed in 10 per cent formalin embedded in paraffin and stained with hematoxylin and eosin

Two animals of each group were tested with carbon for phagocytic activity prior to autopsy Seven rats were injected with saline as controls for the restim injected animals Three of them were tested with carbon 24 hours 48 hours and 5 days after injection while the others (4 rats) were sacrificed 6 days later Lipids—both active (L 92) and inactive—were prepared by V Z Pasternak of our laboratory by organic chemical fractionation procedures of active restim The activity of fractions was assayed by their effect on phagocytosis The inactive lipids used as controls were not exact inactive counterparts of the active fraction Rather they represented alcohol soluble inactive lipids derived from the crude lipid

Ten animals were injected with inactive lipid as controls against the active lipid All animals were examined hematologically prior to injection and directly before autopsy including red count white count and differential

Results of Restim Injection

Hematological Findings

There was no significant fluctuation of the number of red and white cells in peripheral blood There was however a relative increase of neutrophils that

* The work described in this paper was supported in part by grants from the Office of Naval Research and the National Science Foundation Washington D C

throughout the acini Some nodules were directly adjacent to central veins and sometimes projected into the lumen of the latter Perivascular cell hyperplasia in interstitial tissue also was more significant than that of the 48 hour group (FIGURE 2)



FIGURE 2 A part of liver acinus from rat 5 days after injection with restim showing proliferation of Kupffer s cells

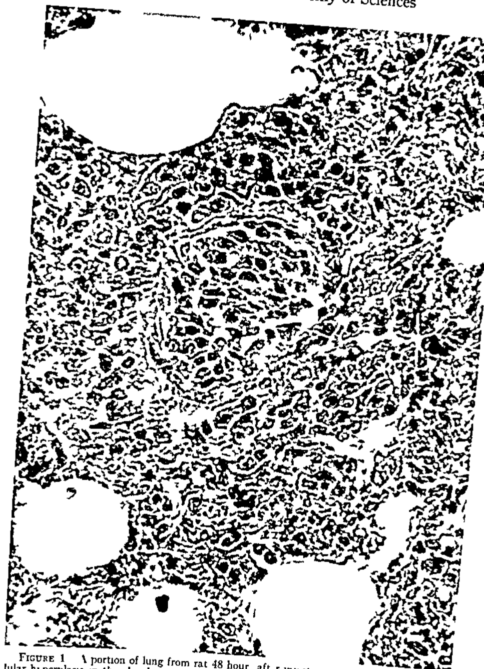


FIGURE 1 A portion of lung from rat 48 hour aft r injecti n with rest m showing cellular hyperplasia in the alveolar septa especially in int rstit al paces urroun ling arteriole (*center*) The lumen of the arteriole is mostly ol lite ate l due to prolif ration of ndothelial cells

and only a slight diminution of perifollicular cell zones could be detected Signs of degeneration of lymphocyte in follicles congestion and sometimes slight infiltration of leukocytes in the red pulp of the spleen could be seen

Five days follicles especially their perifollicular cell zone diminished re



FIGURE 4 Lung from rat 24 hours after injection with the lipid showing the radial arrangement of proliferating adventitial cells of a terioles

Spleen

Twenty four hours there was no significant change in spleen in comparison with that of the control group

Forty eight hours changes of spleen at this stage were not very significant,

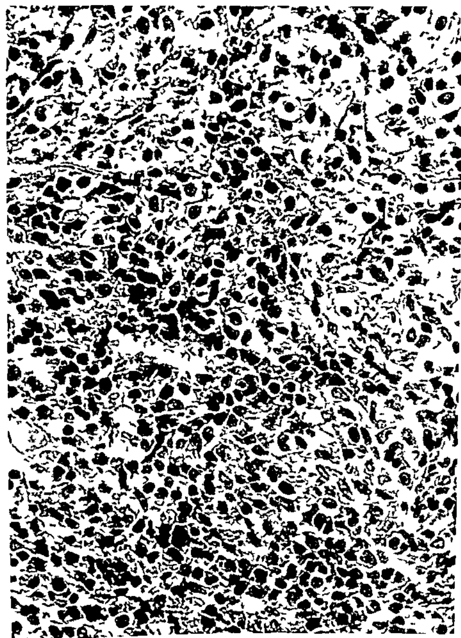


FIGURE 3 Spleen pulp from the rat 5 days after injection with restim illustrating the new growth of reticulum cells which are characterized by small cell bodies and dark nuclei

Part IV The Relations of the Reticuloendothelial System to Stress, Organic Metabolism and Growth Processes

THE CONTRIBUTION OF THE RETICULOENDOTHELIAL SYSTEM TO THE DEVELOPMENT OF TOLERANCE TO EXPERIMENTAL SHOCK*

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Before entering into a discussion of the mechanisms involved in different forms of tolerance to stress and their possible relation to the reticuloendothelial system (RES), it would be useful to catalogue the evidence linking disturbances in RES function to the development of shock. In a recent survey of the phagocytic behavior following protracted hemorrhagic shock in the rat[1] it was shown that RES function became progressively impaired as the shock deepened. Subsequent studies in the rabbit showed a comparable effect coincident with the development of the irreversible stage of hemorrhagic shock. TABLE 1 shows that the phagocytic index K, measured by following the clearance of carbon from the blood stream, remains depressed for at least 24 to 48 hours after exposure to severe hemorrhagic shock.

Conversely, it has been shown that interference with the RES by overloading the phagocytic elements with large amounts of colloid renders the animal highly sensitive to all forms of stress, including hemorrhagic and traumatic shock,[2] infusions of vasoactive amines, bacterial endotoxins,[3] exotoxins of bacterial origin, histamine, and whole body X irradiation.[4] The predisposing action of RES blockade is temporary in nature, lasting from 4 to 12 hours in most cases. The fact that a similar impairment of the capacity to withstand shock and endotoxemia develops in germ free rats that have received blocking doses of colloidal iron would seem to rule out bacterial toxemia as the decisive factor affected by blockade of the RES.[5] The precise mechanism involved must await clarification of the many changes in the blood constituents and in the vessels themselves which have been shown to be activated by the injection of large amounts of colloidal suspensions and their uptake by the RES.

Morphologic evidence also has been provided to show that the Kupffer's cells of the liver and the phagocytes of the spleen undergo degenerative and inflammatory changes following various forms of shock and blockade.[6] Inflammatory changes involving the phagocytes of the liver and spleen are most prominent in animals pretreated with colloidal thorium dioxide (Thorotrast) alone or subjected to shock after blockade with Thorotrast. As may be seen in FIGURE 1, clusters of neutrophils surround the damaged Kupffer's cells in the liver as early as 8 to 10 hours after hemorrhagic shock (B.P. at 40 mm Hg for 3 hours).

There is thus a clear cut history of disturbed reticuloendothelial function

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† Established Investigator of the American Heart Association.

markably, and the boundary line between red and white pulp was not very clear Red pulp of spleen was enlarged mainly by a striking increase of cell elements *in loco* which were characterized by a lymphocytelike size scanty cytoplasm and round nuclei with abundant chromatin (FIGURE 3) On the other hand, fixed type reticulum cells decreased in number

Results of Lipid Injection

Ten white male CFN rats were injected intravenously with about 30 γ of the lipid extracted from restim (L 92) Ten white male CFN rats were injected intravenously with 30 γ of inactive lipid as controls After testing carbon clearance, all animals were autopsied 24 hours later and examined histologically

The histological changes of organs from the L 92 treated rats were essentially the same as those of rats treated with restim at the same time However some differences occurred as follows in the lung (FIGURE 4) cell response tended to be limited only in the perivascular spaces of arterioles where newly growing cells resembling plasmacytes accumulated uniformly Hyperplasia of intimal cells of arterioles was less Although there was slight proliferation of adventitial cells of arterioles in the lung in the controls its grade was much lower and these cells resembled lymphocytes rather than plasmacytes

Activation of Kupffer s cells in liver seemed to begin in 24 hours following injection and the degree of carbon storage was somewhat higher than that of cases treated with inactive lipid

In the spleen there was more intensive phagocytosis of each reticulum cell in red pulp but at 24 hours remarkable growth of reticulum cells was not yet seen

Further studies of comparative morphologic changes due to active lipid at 2 days 5 days 4 weeks and 3 months are now under way These studies will be compared with functional tests of antibody synthesis carried out concurrently

Reference

1 CUTLER J L 1960 The enhancement of hemolysin production in the rat by zymosan J Immunol 84 416

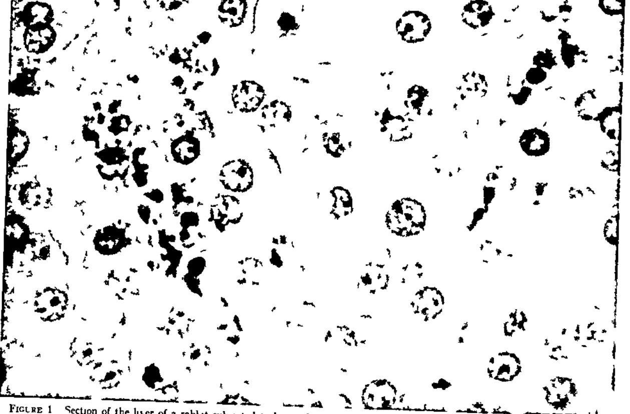


FIGURE 1 Section of the liver of a rabbit subjected to hemorrhagic shock (3 hours at 40 mm Hg) Eight hours after blood replacement the Kupffer's cells show signs of degeneration and local accumulation of neutrophils Parenchymal cells comparatively unchanged Hematoxylin and eosin ×850

coincident with different kinds of stress and evidence that experimental impairment of RES function (at least as concerns phagocytic behavior) interferes markedly with the animal's ability to combat shock.

When laboratory animals are exposed repeatedly to noxious stimuli they become increasingly resistant to the offending agent, provided the episodes are properly spaced and the intensity of the stimulus is graded.[7] Some procedures give rise to an adaptive reaction that is highly specific, while others condition the animal to a much broader spectrum of deleterious phenomena. Thus the literature contains references to induced tolerance to specific chemical poisons,[8] to vasoactive constrictor and dilator substances,[9] to bacterial tissue products[10] and even to normally lethal episodes of trauma.[11] Development of tolerance has been attributed to numerous factors, ranging from specific adaptive mechanisms such as immune phenomena, adaptive enzymes, and hypertrophy of particular cells, to a more efficient utilization of substrates. However, one cannot readily explain the conditioning that leads to resistance to major stresses

TABLE 1
EFFECT OF HEMORRHAGIC SHOCK ON THE RES

	Phagocytic index (*K*)	
	Rat	Rabbit
Control	0.02	0.03
1 Hour hypotension	0.012	0.02
2 Hours hypotension	0.009	0.015
3 Hours hypotension	0.007	0.010
24 Hours later	0.014	0.02

Measured by clearance of carbon 1 hour after blood replacement (average of 6 animals in each group).

such as shock or tissue injury, on the basis of any particular set of circumstances. The fact that one set of stimuli set into motion adaptive measures that were effective against apparently unrelated stimuli raised the possibility that all of these situations eventually involve a common factor or set of factors whose response to repeated stress was essential to the successful development of the conditioned state. The other alternative would be that shock is attended by changes in a great many regulatory mechanisms in an unpredictable manner, depending upon innumerable facets of the host's response and the particular nature of the challenge. Tolerance could then be engendered by an adaptive response in many different homeostatic systems.

Inasmuch as most forms of shock eventually involve deterioration of vascular reactivity at the level of the microcirculation, it has been our thesis that the multiplicity of initiating and sustaining factors must converge and, in the final analysis, operate through some aspect of neurohumoral homeostasis.

Adrenal Hypertrophy

One of the first experimental models used to study the adaptive process was the resistance to lethal traumatic shock engendered by subjecting rats to re

capacity to clear colloid from the blood stream returned to normal thereafter until at 24 hours phagocytic function usually had compensated to above normal levels In the adrenalectomized rat on the other hand the blockade induced by colloid persisted for as long as 48 to 96 hours The administration of blocking doses of colloid was lethal to a large percentage (30 to 40 per cent) of these animal

The functional inadequacy of the RES in adrenalectomized animal became further evident when attempts were made to stimulate the system with repeated injections of graded amounts of colloid Under normal condition animals injected for 4 to 5 days with moderate amounts of colloid showed a much more rapid clearance of labeled particulate matter from the blood stream[18] When this procedure was continued for from 7 to 10 days the size of the liver and spleen increased by as much as 40 to 70 per cent Attempts to stimulate the RES in adrenalectomized rats were unsuccessful when the usual amounts of colloid were used Stimulation could be achieved only when small amounts of colloid were administered and when the conditioning regime was spread out over a period of several weeks

Adrenalectomized animals did not show the same degree of stimulation as did normal control For example K values in a normal rat could be increased almost eightfold (from 0 01 to as high as 0 08) whereas in adrenalectomized rats the K values rarely were increased more than threefold

It was also possible to avoid the toxic effects of repeated doses of colloidal materials in adrenalectomized rats by the following procedure Immediately upon surgical removal of both adrenal supportive cortisone treatment was begun Daily intramuscular injections of 5 mg cortisone were given for a period of 2 weeks As soon as the rats had recovered from the operative procedure a graded series of injections of bacterial endotoxin was begun following the same schedule as in controls for inducing tolerance By the tenth day these adrenalectomized cortisone supported animals had become tolerant to usually lethal amounts of endotoxin The corticosteroid treatment was then discontinued and replaced by a high salt intake Maintenance doses of endotoxin given daily effectively maintained the tolerant state for at least 4 weeks Such animal showed a rapid clearance of carbon ($K = 0\ 05$) and were resistant to Noble Collip drum shock

The adrenal apparently serves an important function in maintaining the conditioned state The RES in adrenalectomized rats lost its stimulated status within 48 hours after the last treatment The presence of the adrenal gland thus permits full development of the potentiality of the RES to undergo hypertrophy and hyperplasia The reticuloendothelial cells have the intrinsic capacity to respond in the absence of the adrenals but only to a limited extent

It has been suggested that the systemic manifestations of blockade of the RES may in part be due to an associated depression of adrenocortical activity In order to test this circumstance rats were given 5 mg cortisone/100 gm body weight for 2 to 3 days preceding blockade together with a dose of cortisone at the time of injection of the blocking colloid The associated predisposing action of blockade was still present in these animal as tested by the failure to withstand normally tolerated amounts of drum trauma

peated tumbling in the Noble Collip drum[7] Examination of biochemical changes in the blood and tissues offered no ready explanation for the phenomenon[1] In an effort to determine whether any particular organ system was involved, a survey was made of the histological characteristics of the tissues of such animals Two organs the liver and the adrenals, showed striking changes The Kupffer s cells of the liver were unusually prominent and appeared to have increased in number the adrenals were significantly enlarged and gave the appearance of that observed in animals that had been stimulated by repeated injections of ACTH On this basis it was speculated that repeated exposure to trauma caused the release of ACTH stimulation thereby of the adrenal cortex and in turn hypertrophy of the RES

Experiments therefore were set up to reproduce this circumstance by injecting ACTH on the same time schedule as the repeated trauma used to induce resistance in rats The rats were treated with ACTH (10 mg Acthar*) for 8 days and then exposed either to drum shock or to lethal amounts of bacterial endotoxin (*Escherichia coli* lipopolysaccharide) These treated animals were not significantly more resistant either to drum shock (650 turns) or acute endotoxemia (2 0 mg /100 gm) Histological inspection of the liver verified the fact that the treatment with ACTH had caused the Kupffer s cells to become considerably hypertrophied Thus although it was possible to reproduce the stimulation of the RES by adrenal intervention the resistance to stress was not duplicated by this hormonal mechanism alone Apparently physical hypertrophy of the RES does not by itself represent a key process in the adaptation to trauma

Tolerance in Adrenalectomized Rats

Since there was so much evidence suggesting that the adrenals were implicated in some aspect of the conditioning process a series of experiments was instituted to circumscribe more specifically the relationship of the adrenal both to stimulation of the RES per se and to tolerance in general It is known that adrenalectomized animals are highly susceptible to shock[13] and to bacterial endotoxemia[14] This heightened susceptibility can be demonstrated even in adrenalectomized rats maintained for indefinite periods in excellent health without supportive hormone treatment by a high salt intake It was therefore of interest to determine whether this susceptibility was associated with a concomitant suppression of the RES

The phagocytic behavior of the reticuloendothelial cells in the e animals was examined by the carbon clearance technique of Benacerraf and Halpern[15] The rate of removal of colloidal carbon (phagocytic index K) from the blood stream of adrenalectomized self maintained rats was essentially the same as that of normal controls Further studies however revealed that the RES was subnormal in particular by attempting to alter the functional capacity of the system both in the direction of stimulation and blockade Thus in normal rats the injection of substantial amount of colloidal material (carbon saccharated iron or Thorotrast) had a transient depressing action on the phagocytic activity of the RES lasting for from 4 to 8 hours depending on the dose The

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that other facets of RES functional activity had been augmented. In the experiments with ACTH, morphologic hypertrophy of the RES was not associated with resistance to endotoxin or shock. Similarly, resistance to drum trauma was not necessarily associated with a hypertrophied appearance of the reticuloendothelial cell. As indicated below, certain materials are capable of stimulating particular enzyme functions of the RES in addition to their effects on phagocytic activity and cell hypertrophy.

Vascular Effects of Modifications in the RES

It has been assumed in the past that stimulation of the RES primarily involved some facet of the phagocytic properties of this system. Evidence has also been accumulated to indicate that the uptake of colloid by the cells of the RES is associated with changes in endothelium in other parts of the body[17] and in the reactivity of vascular smooth muscle.[18] Two experimental procedures were used: direct observational measurements on the microcirculation and perfusion of isolated vascular beds.

Studies on the mesoappendix of the rat indicated that blockade of the RES was associated with an increased reactivity of vascular smooth muscle. An enhanced response was obtained not only with topically applied catecholamines but with dilator substances such as histamine. The vascular effects were present only during the period of blockade, since reactivity values returned to normal after 12 hours. In addition to the effect on vascular smooth muscle, the capillary endothelium of blockaded animals was found to be unusually susceptible to damage by microtrauma and by the local application of large doses of epinephrine with micropipets. In fact, even minor manipulation of the capillary vessel with microneedles resulted in damage, as manifested by the rapid accumulation of carbon from the blood stream onto the capillary wall.

Experiments were also carried out in blockaded animals in which epinephrine (5 to 10 μg/min) was infused continuously for several hours. Under these circumstances the major part of the microcirculation shut down and failed to reopen even after the infusion had been terminated. Apparently, interference with the reticuloendothelial cell either removed some substance from the blood or added a vasotoxic factor. This deficiency was most striking in blockaded animals that subsequently received an intravenous injection of bacterial endotoxin. Under these conditions the terminal arterioles, precapillaries, and venules became markedly dilated and large areas of capillary stasis developed with unusual rapidity.

It was possible to demonstrate that the effect on the small blood vessels was an intrinsic defect and due not merely to some change in the makeup of the circulating blood by isolating an appendage, such as the ear, from a Proferrin or Thorotrast blockaded rabbit and studying its response under *in vitro* conditions. In contrast to controls, such perfused preparations were unstable and became edematous within 5 to 10 min under perfusion pressures that normally sustain the flow through the ear at normal levels for at least several hours. The abnormal state of the vascular endothelium in the perfused tissue was also shown by adding colloidal carbon to a perfusate. Unlike the ear from normal

The adaptive response to repeated stress has been assumed by some investigators to be related to adrenal hyperfunction. As indicated above with reference to RES stimulation, adrenalectomized rats were capable of developing tolerance to drum trauma and to bacterial endotoxins by a carefully graded regime. In both instances the tolerance developed was such that the conditioned adrenalectomized animal was definitely more resistant than its adrenalectomized untreated counterpart, but was not equivalent to the spectacular increase in resistance produced in normal controls with intact adrenals (TABLE 2).

As in controls, conditioning to endotoxin was associated with a stimulation of the RES. The stimulated state persisted for approximately 24 to 48 hours after completion of the conditioning regime. The injection of blocking doses of saccharated iron oxide (Proferrin) or Thorotrast in adrenalectomized conditioned rats completely abolished the resistance but, unlike the situation in control animals, there was no return of the resistant state. In fact, previously

TABLE 2
CONDITIONING TO DRUM TRAUMA IN ADRENALECTOMIZED RATS

	Interval after last priming	
	12 hours	72 hours
Controls—saline	8/10	8/10
Controls—drum resistance	10/10	10/10
Adrenalectomized—saline	1/10	0/10
Adrenalectomized—drum resistance	8/10	1/10
Adrenalectomized + cortisone—drum resistance	9/10	3/10

Survival after 600 turns in Noble Collip drum.

tolerant adrenalectomized animals usually succumbed within 4 to 5 days after blockade of the RES.

RES Stimulation and Tolerance

In a further study of the mechanisms involved in resistance, attempts were made to suppress the resistant state in conditioned animals by treating them with large doses of cortisone and whole body X irradiation. These agencies were selected since it was known that under normal circumstances treatment with large doses (25 mg.) of cortisone or whole body X irradiation (400 to 600 r) led to an increased susceptibility to trauma.[2] It was found, however, that such treatment after the induction of resistance did not abolish the conditioning effect. Apparently these agencies act by interfering with the capacity of the RES to be stimulated rather than by a direct cytotoxic effect. Thus, once resistance has been established, exposure to X rays or cortisone did not alter the adaptive mechanisms set into play by the already stimulated and hypertrophied RES.

As indicated previously, the fact that the phagocytic properties of the RES were enhanced by a given experimental condition did not necessarily imply

state of tolerance As indicated none of the conditioning measures results in a permanent resistance Unless the animals are repeatedly exposed to the conditioning stimulus tolerance disappear However it was found possible to sustain one form of adaptation such as drum trauma by exposing the animal to another agent bacterial endotoxin The converse situation tolerance to bacterial endotoxin also could be sustained by exposing the animal to drum trauma (TABLE 3) Evidently once the conditioning process has been initiated other factors can sustain the effect

Immunological Aspects of Tolerance

There is suggestive evidence that the adaptive response to stress may involve an immunological reaction[11] Several investigators have pointed out that bacterial endotoxins give rise not only to specific antibodies but also to heterologous antibodies Thus a broad spectrum of resistance is induced to different biological substances Effects similar to those produced by bacterial endotoxins were evoked by extracts of normal tissues Therefore the possibility exists that repeated injury to tissue may give rise to the release of tissue products such as polysaccharides that stimulate the production of antibodies with broad cross reaction As reported elsewhere in this monograph the RES may be concerned with the production of certain globulins or antibodies Passive transfer experiments have not been sufficiently conclusive to support this interpretation

The adaptive reaction also may involve immune type phenomena within the tissues themselves The RES in addition to its possible function in the production of antibodies may be concerned with the production of some cofactor necessary for antigen antibody interaction These possibilities however must remain speculative until substantiated by more definitive experimental data

References

1 ZWEIFACH B W & B BENACERRAF 1958 Effect of hemorrhagic shock on the phagocytic function of Kupffer cell Circulation Research 6 83

2 MCKENNA J M & B W ZWEIFACH 1956 Reticulo-endothelial system in relation to drum shock Am J Physiol 187 263

3 BEESON P B 1947 Effect of reticulo endothelial blockade on immunity to Shwartzman phenomenon Proc Soc Exptl Biol Med 64 146

4 BENACERRAF B I K ROSENBERG M M SEBESTYEN & B W ZWEIFACH 1959 The effect of high doses of x irradiation on the phagocytic proliferative and metabolic properties of the reticulo endothelial system J Exptl Med 110 49

5 ZWEIFACH B W H A GORDON M WAGNER & J A REYNIERS 1958 Irreversible shock in germfree rats J Exptl Med 107 437

6 MCCLUSKEY R T B W ZWEIFACH W ANTOPOL B BENACERRAF & A L NAGLER Pathogenesis of shock I Absence of morphologic evidence for the presence of bacterial endotoxin Am J Pathol In press

7 NOBLE R 1953 Development of resistance by rats and guinea pigs to amounts of trauma usually fatal Am J Physiol 138 346

8 BURROWS H 1932 Some Factors in the Localization of Disease in the Body Bailliere Tindall and Cox London England

9 FRIESEN H L 1952 Further observations on certain responses of tolerant and control animals to massive doses of epinephrine Am J Phys 171 78

10 GREEN H N & H B STONER 1950 Biological Actions of the Adenine Nucleotides Lewis London England

11 ZWEIFACH B W 1958 Microcirculatory derangements as a basis for the lethal manifestations of experimental shock Brit J Anesthesiol 30 466

rabbits such vessels became coated with carbon Histological sections of the ear showed the carbon particles to be localized primarily in the endothelium of the venules and small veins

Relation of the RES to Vasoactive Chemical Mediators

Adaptive reactions within the tissue proper related either to the formation handling or presence of local tissue mediators also have been suggested to account for the altered status of the vascular system in conditioned animals[19] Conceivably repeated exposure to trauma or the repeated injection of colloids might serve to release vasoactive substances from their storage sites in the tissues and thus act in the same way as histamine depleting agents

A series of experiments was conducted with a variety of histamine releasers in both the rat and the rabbit Despite the fact that the phagocytic activity

TABLE 3
CAPACITY TO SUSTAIN SPECIFIC AND CROSS TOLERANCE

Co d t g g nt	S rv l ft dd t ld ly t atm t f 2 we k		
	S l e (1 ml)	E d t (50 μg)	Drum t m (300 t)
	Resistance to drum trauma		
Endotoxin tolerant rats	2/10	9/10	10/10
Drum resistant rats	4/10	10/10	9/10
	Susceptibility to endotoxin†		
Endotoxin tolerant rats	3/10	10/10	9/10
Drum resistant rats	1/10	/10	2/10

Challenged by tumbling 850 turns in Noble Collip drum
† Challenged with *E coli* lipopolysaccharide (3 mg)

of the RES was unaltered in all of these circumstances the animals consistently showed an enhanced resistance to both traumatic shock and bacterial endo toxemia[0] It is significant to note that this form of adaptation al o could be circumvented by blockade of the RES

The data dealing with the effect of amine relea ers suggests that conditioning involves at least two separate mechanisms the first associated with the RES the second with peripheral factors involving local tissue mediators The fact that blockade of the RES al o aborts the resistance engendered by treatment with amine releasers favors the concept that the vasotoxic effect of such amines may be dependent upon some factor produced by the RES This interpreta tion is supported by the extensive tissue damage produced by epinephrine infusions in RES blockaded animals Io sibly the activation of blood proteo lytic enzyme systems by blockade of the RES may be involved

Cross Tolerance

The basic importance of the RES in adaptive mea ures leading to tolerance is strengthened by experiments concerned with the maintenance of a given

HUMORAL MODIFICATION OF THE FUNCTION OF THE RETICULOENDOTHELIAL SYSTEM

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Introduction

Although the state of shock has been the subject of innumerable investigations, the basic physiological alterations leading to circulatory collapse during shock remain evasive. Recent evidence indicates that the reticuloendothelial system (RES) may play an important role in these homeostatic adjustments.

Suppressing the function of the RES through blockade with various colloidal agents has been shown to lower the capacity of animals to withstand the effects of endotoxins[1,2] or of traumatic and hemorrhagic shock.[3,4] Moreover, resistance acquired against the lethal effects of these stresses can be overcome by blocking the RES.[5-7] Significant alterations in the macrophagic elements of the spleen, liver, and lymphoid tissue have been observed in profound shock, and it is suggested that changes in these bacterial defense mechanisms are important features in overcoming the effects of this injury.[8-10] In addition, stimulation of the RES activity has been observed in certain instances to increase the tolerance against the shock state.[5,11]

The present studies have attempted to understand more clearly some aspects of the function of the RES in resistance against traumatic shock. A humoral mechanism capable of modifying RES function is indicated. Previous work has been reported elsewhere.[12]

Materials and Methods

Young adult rats of the CF Nelson strain (140 to 175 gm.) raised under pathogen free conditions in the Walter Reed Army Institute of Research colony[13] were used in these experiments. A standardized form of traumatic shock was administered by the method of Noble and Collip.[14] Each animal was placed in a revolving circular drum with a diameter of 15 inches and a depth of 8 inches and 2 blunt triangular projections 2 inches high and 3 inches at the base. As the drum revolves at 40 rpm, the animal is carried up the side on one projection. It is then dropped and picked up by the following projection. To prevent rats from jumping over the projections, their hind paws were taped together. The tumbling apparatus † was especially built to accommodate as many as 18 rats (FIGURE 1), allowing large numbers of animals to be tumbled under identical conditions.

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† Property of the Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

12 TOBY C G & R L NOBLE 1944 Factors affecting the development of resistance to traumatic shock in the rat Can J Research 22(Sect E) 79
13 SWINGLE W W & J W REMINGTON 1944 The role of the adrenal cortex in physiological processes Physiol Revs 24 89
14 HARTMANN F A & W J M SCOTT 1932 The protection of adrenalectomized animals against bacterial intoxication by extracts of adrenal cortex J Exptl Med 55 63
15 BIOZZI G B BENACERRAF & B N HALPERN 1953 Quantitative study of granulopectic activity of the reticulo endothelial system II A study of the kinetics of granulopectic activity of the RES in relation to the dose of carbon injected Brit J Exptl Pathol 34 441
16 ZWEIFACH B W B BENACERRAF & L THOMAS 1957 The relationship between the vascular manifestations of shock produced by endotoxin trauma and hemorrhage II The possible role of the reticulo endothelial system in resistance to each type of shock J Exptl Med 106 403
17 BENACERRAF B R T MCCLUSKEY & D PATRAS 1959 Localization of colloidal substances in vascular endothelium A mechanism of tissue damage I Factors causing the pathologic deposition of colloidal carbon Am J Pathol 35 75
18 ZWEIFACH B W & L THOMAS 1957 The relationship between vascular manifestations of shock produced by endotoxin trauma and hemorrhage I Certain similarities between the reactions in normal and endotoxin tolerant rats J Exptl Med 106 385
19 LILLEHEI R C & L D MACLEAN 1959 Increased resistance to hemorrhagic shock in epinephrine tolerant dogs Federation Proc 18 415
20 ZWEIFACH B W & A L NAGLER Tolerance to experimental shock in animals conditioned with amine releasing agents Am J Physiol In press
21 MORGAN H R 1948 Tolerance to the toxic action of somatic antigens of enteric bacteria J Immunol 59 129

heparinized (2 mg /10 ml blood), and centrifuged at 4 C The pooled plasmas were brought to a *p*H of 5.5 by the addition of 1 N HCl boiled for 10 min and filtered The pleens and livers were collected and homogenized in a Waring Blendor at 4° C Acid saline (*p*H 5 5) was added (5 ml /gm of spleen 2 ml /3 gm of liver) during homogenization and the material was acidified to a *p*H of 5 5 by the addition of 1 N HCl These homogenates were then boiled for 10 min and filtered All extracts were stored at 4° C when not u ed and brought to room temperature just before injection Recipient animal (adult female CF Nelson rats 140 to 175 gm) were employed to determine the protective effect of the acidified boiled extracts Each animal received 4 subcutaneous injection during a period of 36 to 48 hours of the following volumes 1 ml of pla ma extract equivalent to approximately 3 ml of original whole plasma (a total equivalent of 12 ml of original plasma) 1 5 ml of spleen extract equivalent to approximately 0 38 gm of ti ue (a total equivalent of 1 5 gm or 2 7 spleens) and 1 5 ml of liver extract equivalent to approximately 3 gm of ti ue (a total equivalent of 12 gm or 2 livers) Control received equal volume of acid saline Three and one half hours after the last injection all animal were subjected to 725 turns in the tumbling apparatus

Splenectomies and bilateral adrenalectomies were performed under light ether anesthesia Animal were checked at autop y to determine completeness of the operations Drinking water of the adrenalectomized rats consisted of 1 per cent saline In replacement studies animals received 1 5 mg corti one in 0 1 ml physiological saline for 2 days On the third day the animals were adrenalectomized and continued on similar do es of hormone 5 times over the subsequent 48 hours Control animal underwent ham operation and received similar volumes of physiological saline All animals were then subjected to 650 rotations in the Noble Collip drum and survival was determined

Survival data were analyzed statistically by the chi quare test and the levels of significance are indicated by probability values (*p*) in the tables

Survival from Drum Trauma

The results shown in FIGURE 2 parallel those of Noble and Collip[14] relating the number of rotations in the drum to the percentage survival No deaths were observed when rats were tumbled for less than 400 rotations However beginning at 500 revolutions the number of animals dying is proportional to the number of revolutions in the drum Accompanying this increased mortality at higher revolutions is a hortened survival time after trauma All animal usually succumbed by 6 hours after drumming At 800 turns in the drum all rats died of shock many even before removal from the drums It is seen from FIGURE 2 that 650 rotations yield a survival of 70 per cent a convenient level to observe a lowering of re istance whereas 750 rotations yield a survival of 30 per cent a good base level to observe an increase in resistance

Many factors have been ob erved to alter the survival of rats subjected to drum trauma We have previously de cribed difference due to ex and striking shifts during different ea ons of the year[12] Males are le s resi tant than female to this type of stress roughly by 100 turns During the summer and fall 30 per cent survival was obtained with 750 turns but this ro e to 45 per cent during the winter months and dropped as low as 10 per cent during the

Impairment of the phagocytic activity of the RES was produced by blockading the reticuloendothelial elements with colloidal agents. This method physically overloads the phagocytic cells of the liver and spleen so that no further uptake occurs during a distinct time interval.[3,15,17] In the present studies animals were injected via the jugular vein with 77 mg (in 0.3 ml volume) of Thorotrast, a stabilized colloidal suspension of thorium dioxide and with 12 mg (in 0.6-ml volume) of Proferrin, a saccharated iron oxide

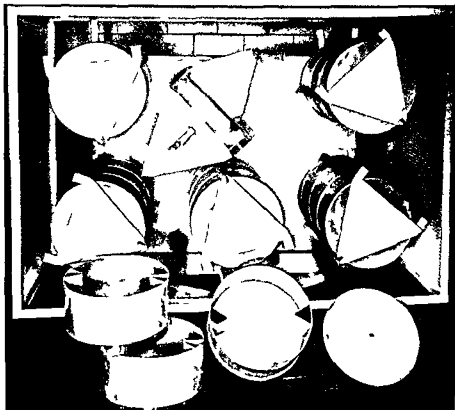


FIGURE 1. Modified Noble Collip tumbling apparatus for the production of drum trauma in rats. Eighteen drums in stacks of 3 are rotated simultaneously at 40 rpm. Reproduced from *Reticuloendothelial Structure and Function*. John H. Heller, Ed. By permission of The Ronald Press Company.

Control animals received an equal volume of physiological saline by the same route. Two hours after administration of the colloidal agents, animals were subjected to trauma in the Noble Collip drum and survival was determined.

Resistance to traumatic shock was attained by subjecting rats to repeated sublethal episodes of drum trauma as outlined above. Various tissues and plasma were extracted from these trauma-resistant rats in an attempt to isolate an active factor that was responsible for this induced state of tolerance. These preparations were then administered to normal animals and their beneficial effects were assessed. Blood was removed from donor rats by cardiac puncture

and heart beat are increased and diarrhea is frequently present Hemoconcentration and loss of plasma volume accompanied by a drop in body temperature regularly occur[14, 22, 23] Blood pressure drops immediately after severe doses of trauma[22] but shows an initial rise after sublethal trauma (R L Griswold personal communication) Although there have been no systematic studies of the pathological changes involved several investigators have noted the extreme vascular engorgement of the abdominal viscera[14, 19, 27, 28]

About 10 per cent of the animals die of acute injury in the drum usually from a ruptured spleen or rarely from subarachnoid hemmorrhage and are not routinely included in the tabulation of results

Biochemical studies of blood and tissues of traumatized rats show that several important changes occur[25, 26, 27] Significant increases in blood glucose and nonprotein nitrogen (NPN) become apparent while glycogen stores are depleted Lactate and pyruvate levels rise and the lactate pyruvate ratio is increased indicating a change from aerobic to anaerobic metabolic pathways Adenosine triphosphate (ATP) and phosphocreatine levels are lowered while phosphate pentose uric acid creatine and creatinine are increased Lacker *et al*,[23] observed an impaired phosphorylation in mitochondria isolated from heart muscle of traumatized rats A decreased synthesis of ATP was also observed but no changes in the levels of respiratory pigments or ATPase activity were found

It is clear that with this experimental procedure trauma can be applied in a graded controlled manner Following this insult a typical picture of shock develops that is uncomplicated by infection hemorrhage or anesthesia Thus drum trauma elicits a reproducible form of body injury and seems appropriate for testing the relative effectiveness of certain injurious or protective procedures

Conditioning of Animals to Trauma

Perhaps the most interesting and most useful observation in traumatic shock is the phenomenon of conditioning Noble and his associates[25, 9] observed that animals acquired resistance to drum trauma when subjected to repeated sublethal doses of this stress and they could then withstand a degree of trauma that was otherwise fatal The rats were subjected to increasing rotations every other day beginning with 200 turns the first day By day 14 they received 1000 turns without mortality Toby and Noble[13] subsequently showed that a single exposure was sufficient to cause resistance but it lasted for only a few days

Since we required large numbers of trauma resistant animals we shortened this regime considerably without affecting the degree of tolerance Animals were subjected to 200 turns on days 1 and 2 and 300 turns on days 3 and 4 On day 5 these animals were found to be trauma resistant and withstood 900 rotations without mortality (TABLE 1) As seen from the data it was possible progressively to raise the number of rotations to 2500 turns with only small mortality resulting principally from mechanical injury These resistant animals maintained their resistant state for more than 2 months

The conditioned animal appears normal after exposure to otherwise lethal doses of drum trauma It responds to normal stimuli does not drink exces

spring before returning to about 30 per cent in June Certain species differences have also been observed J McLaughlin, Jr, for example has observed a high percentage of mortality during midsummer for Sprague Dawley rats (personal communication) McLaughlin has also observed a consistent decrease in mortality in rats tumbled in the afternoon as opposed to the morning and in rats that were starved for 18 hours He suggests that, since rats are

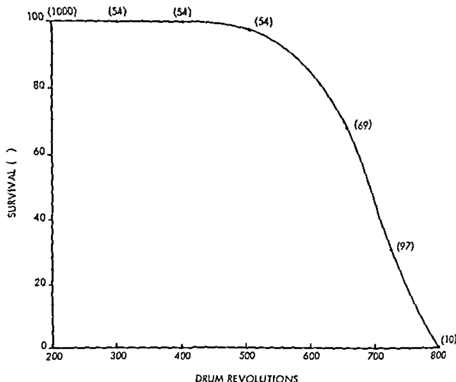


FIGURE 2 Per cent survival in female rats following graded degrees of trauma The number of animals in each point is indicated in parentheses Reproduced from *Reticuloendothelial Structure and Function* John H Heller Ed by permission of The Ronald Press Company [12]

nocturnal animals and eat during the night their semistarved condition may protect them when tumbled in the afternoon

Other phenomena are known to affect the susceptibility and resistance to drum trauma especially prior exposure to various forms of stress [14 18 21] Some of these will be described in the following sections All of these must be considered in evaluating subsequent experiment and control animals must be studied concurrently

Traumatic Shock in Normal Animals

Symptoms of shock rapidly appear in the rats after they are removed from the drum The animals are quiet but respond to strong stimuli respiration

days 3 and 4 Twenty four hours after the last drumming these trauma resistant animals were injected as above with either colloidal agent or saline and tested 2 hours later at 900 rotation As seen in TABLE 3 this type of RES blockade overcomes the resistance of trauma adapted animals Untreated trauma resistant animals and those receiving saline injections withstood 900 rotations without mortality whereas approximately 50 per cent of the trauma resistant rats receiving Thorotrast or Profermin succumbed

These results agree essentially with those of McKenna and Zweifach[3] and Zweifach and Thomas[4] who further observed that other colloidal preparations were equally effective in undermining the capacity of rats to withstand traumatic shock These investigators found this effect to be time-dependent becoming evident within 1 to 2 hours and lasting for 4 to 5 hours after injection This corresponds to the time intervals of impaired phagocytic activity following blockade

The demonstration that blockade of the RES interferes with the ability of normal animals to withstand trauma and depresses the previously established

TABLE 3
RES BLOCKADE AND SURVIVAL OF TRAUMA RESISTANT (TR) RATS TO DRUM TRAUMA

Treatment	Number of rats	Survival
TR Controls	36	36 (100%)
TR Profermin	36	19 (53%)†
TR Thorotrast	18	8 (45%)†
TR Saline	14	14 (100%)

Rats received 900 turns in Noble Collip drum
† P = <0 001

resistance of trauma adapted animals suggests that such resistance may represent an expression of some function of the RES During conditioning the RES might elaborate or concentrate some material that prevents the manifestations of shock The isolation and subsequent transfer of this substance to normal animals might also transfer this resistance to normal animals and consequently prevent the adverse sequelae of shock

1 *Trauma Protecting Factor*

Various reticuloendothelial organs and the plasma from resistant animals were extracted chemically in an attempt to isolate an active protective principle The extraction procedure was similar to that previously described for obtaining an erythropoietic stimulating material in the blood and urine of anoxic rodents[30,31] The characteristics of this acidified heat stable fraction of plasma have been described[32,33]

Several groups of animals were made resistant to drum trauma by subjecting them to 200 rotations on days 1 and 2 and 300 rotations on days 3 and 4 Twenty four hours after the last drumming these rats were sacrificed and extracts were prepared from their tissues and plasma These preparations were then administered to normal recipient rats over a period of 36 to 48 hours

sively and does not develop diarrhea The circulatory picture remains normal and no sign of congestion appears in the abdominal organs or gastrointestinal tract [14] No increases in blood NPN and pyruvate are observed in the resistant rat and there are only slight rises in phosphate glucose pentose amino acids, and lactate There is no fall in liver glycogen and only slight changes in ATP, phosphocreatine and phosphopyruvate occur [25,27]

Apparently the resistant animal is capable of maintaining homeostasis by stabilizing its metabolic processes, whereas the untrained animal cannot do this

TABLE 1

EFFECT OF CONDITIONING ON SURVIVAL OF RATS TO DRUM TRAUMA

Day	Revolutions	Mortality
1 2	200	0/18
3 4	300	0/18
5†	900	0/18
7	1100	0/18
9	1300	1/18 (6%)
12	1500	2/17 (12%)
15	1700	1/15 ([illegible]%)
18	2000	2/14 (14%)
21	2500	3/12 (25%)
80	900	0/9

No of rats succumbed/total no of rats tested

† On day 5 animals are resistant to 900 revolution a dose fatal to normal animals

TABLE 2

RES BLOCKADE AND SURVIVAL OF NORMAL RATS TO DRUM TRAUMA

Treatment	Number of rats	Survival
Controls	35	23 (66%)
Proferrin	44	12 (27%)†
Thorotrast	18	4 (22%)†
Saline	34	24 (71%)

* Rats received 6 0 turns in Noble Collip drum

† P = <0 01

Interference with RES Function

The participation of the RES in the phenomenon of drum trauma was demonstrated by suppressing the phagocytic activity of the RE elements by blockade Thorotrast and Proferrin were injected intravenously into normal and resistant animals After 2 hours tolerance to drum trauma was assessed by subjecting the e animals to 650 turn in the Noble Collip drum The data in TABLE 2 shows that blockade of the RES with either colloidal agent significantly decreased the survival of normal animal subjected to drum trauma Control animals receiving equal volumes of saline were unaffected Other groups were employed to test the effect of RES impairment on trauma resistant animals Rats were conditioned by rotating 200 turns on days 1 and 2 and 300 turns on

although it is equally possible that this material is not normally present being produced or concentrated during trauma A somewhat analogous condition appears to exist in the endotoxin resistant animal in that serum or plasma from normal animals was observed to interact with bacterial endotoxins forming a product that augments the febrile response [34] Under similar conditions serum from animals rendered tolerant to endotoxins by their daily injection has the ability to inhibit the pyrogenic action of endotoxins Farr and his associates[34] believe that humoral inhibitors, although present in small amounts in normal rabbits develop to a greater extent in the endotoxin resistant animal and account for this state of tolerance

Injection of tissue homogenates and of whole plasma from normal and trauma resistant rats were likewise without effect (TABLE 7) It is of interest that whole plasma from trauma resistant rats did not alter significantly the survival of normal animals to trauma whereas concentrated boiled filtrates of acidified plasma did show a striking protective action This may explain previous failures to protect normal animals against traumatic shock by ad

TABLE 7
SURVIVAL OF NORMAL RATS FOLLOWING ADMINISTRATION OF WHOLE PLASMA AND SPLEEN HOMOGENATES

Treatment	Number of rats	Survival
Normal controls	18	13 (36%)
Physiological saline	12	4 (33%)
Normal whole plasma	4	9 (38%)
TR Whole plasma	24	8 (33%)
Normal spleen homogenate	9	3 (33%)
TR Spleen homogenate	8	2 (25%)

Rats received 725 turns in Noble Collip drum

ministering serum obtained from conditioned animals [15] Ungar,[23] however observed that the injection of untreated serum from previously traumatized animals protected normal animals against trauma He traumatized skeletal muscle by dropping a metal rod onto it from a given height or by shooting small steel projectiles through the adductor region of the thigh Thus direct injury to the muscle tissue may cause greater concentrations of the protective material to be released into the plasma

The protective activity of the humoral factor seems to be inhibited by certain heat labile acid coagulable components normally present in the plasma A similar inhibition of endotoxins by heat labile components of serum also has been observed Thus incubation of bacterial or mammalian polysaccharides with fresh serum but not with heated serum altered the endotoxin materials so they could no longer evoke the characteristic host responses [36] The possibility remains however that the extraction procedure merely concentrates the effective principle and that greater volumes of whole plasma or serum might be equally effective This is difficult to determine since the large fluid volume might also afford protection thus masking the specific action of the humoral factor It was shown for example that fluid replacement with serum plasma

Three and one half hours after the fourth injection the animals were exposed to 725 turns in the tumbling apparatus

TABLE 4 shows that the administration of acidified boiled filtrates of spleen and of plasma from trauma resistant rats significantly increased the survival *of normal rats when injected into these animals prior to trauma* *The adminis*

TABLE 4

SURVIVAL OF NORMAL RATS GIVEN EXTRACTS OF BLOOD AND ORGANS FROM TRAUMA RESISTANT (TR) RATS*

Treatment	Number of rats	Survival
Controls	125	37 (30%)
Acid saline	54	16 (30%)
TR plasma extract	73	54 (74%)†
TR spleen extract	78	55 (71%)†
TR liver extract	37	10 (27%)

Rats received 725 turns in Noble Collip drum
† P = <0 001

TABLE 5

SEASONAL VARIATIONS IN PROTECTION AGAINST DRUM TRAUMA

Treatment	Number of rats	Survival
A Control	36	11 (31%)
TR spleen extract	*36*	*25 (69%)*†
B Control	17	7 (41%)
TR spleen extract	14	13 (93%)†
C Control	43	8 (19%)
TR spleen extract	40	16 (40%)†

* Rats received 725 turns in Noble Collip drum A summer B winter C spring
† P = 0 01

TABLE 6

SURVIVAL OF NORMAL RATS GIVEN EXTRACTS OF BLOOD AND ORGANS FROM NORMAL RATS

Treatment	Number of rats	Survival
Normal spleen extract	95	36 (38%)
Normal plasma extract	37	12 (32%)
Acid saline	17	5 (29%)

Rats received 725 turns in Noble Collip drum

tration of similar extracts of liver from trauma resistant rats or equivalent quantities of acid saline had no effect on survival In spite of the seasonal variations in survival the protective action of the splenic extracts was maintained throughout the year (TABLE 5)

The administration of acidified boiled filtrates of spleen and of plasma from normal untraumatized animals (TABLE 6) did not produce any significant protecting action Greater concentrations or more specific extraction methods of these normal tissues may reveal the presence of this humoral principle(s)

by boiling[47] and therefore cannot be a factor in the present acidified boiled filtrate Toro[48] has obtained an extract from liver parenchyma that influence the function of the RES

In addition several serum components have been observed to be important for RES function The proliferating and phagocytic capacities of the RES were observed to be dependent on special humoral factors[49,50] and a heat stable serum principle has recently been found necessary for intracellular killing of phagocytized bacteria[51] Elsewhere in this monograph Freedman[52] and previously Farr[53] have reported the passive transfer of tolerance to the toxic effects of endotoxin by plasma of tolerant animals This was correlated with a stimulation of RES function in the recipient animal[5,53] as shown by the accelerated clearance of carbon from the blood similar to the increased clearance seen in animals rendered tolerant by repeated daily injections of endotoxin[4] An augmented phagocytic activity measured either by the rate of carbon clearance[5] or by the uptake of radioactive colloidal gold (Reichard unpublished data) has not been observed in the trauma resistant rat

The suggestion has been made that serum properdin levels are important in resistance or susceptibility of animals to stresses including drum shock[54,55] However it does not seem to be a factor in the present experiments since the preparation of the extracts involved boiling for 10 min a process that destroys properdin[54]

Drum Trauma and Other Stresses

A striking similarity exists between the processes evoked by drum trauma and those involved in poisoning by bacterial endotoxins Besides the resemblance of vascular derangements resulting from lethal amounts of endotoxin to those observed during traumatic and hemorrhagic shock[10] parallel alterations follow RES impairment For example the susceptibility of normal animals to the lethal action of endotoxins is greatly increased following the injection of reticuloendothelial blockading agents such as Thorotrast Proferrin carbon and trypan blue[1,2] In addition a state of resistance can be induced against the toxic effects of endotoxins by repeated daily injections and like the tolerance produced by drum trauma it is eliminated by blockade of the RES[6,7] It is significant that resistance to otherwise lethal exposures to drum trauma or hemorrhagic shock was shown to occur following repeated daily injections of sublethal doses of endotoxin[10,57] Trauma resistant rats were also reported to be more refractory to hemorrhagic shock[58] although they failed to show an increased tolerance against lethal doses of endotoxin[10]

Fine and his associates[9,11] have postulated that irreversibility in hemorrhagic shock is caused in fact by the presence of enteric bacteria or their products in the blood stream They believe that endotoxins are constantly available and readily produce vascular collapse when the endotoxin detoxifying potential of the RES is impaired by shock These investigators reported the presence of a toxic factor that resembles bacterial endotoxin in the blood of animals in hemorrhagic shock Administration of this shock plasma but not of normal plasma produces many of the effects of endotoxin including resistance to hemorrhagic shock when given intravenously for 5 to 6 days to normal rabbits

or isotonic saline solution can protect against fatal burn, hemorrhage, and tourniquet shock when given intravenously, intraperitoneally, or orally following exposure to injury.[37,39] Subcutaneous administration of saline prevented hemorrhagic shock in dogs and mice, experimental wounds in goats, and tumbling shock in rats only when combined with hyaluronidase.[39] Protection by fluid replacement is not involved in the experiments reported here, since the volumes of plasma administered are less than those required for effective therapy. Moreover, plasma was given prior to trauma, it was administered subcutaneously, and control animals that received equal amounts of saline were not protected.

No indication of a toxic substance has been observed. Neither tissue homogenates, whole plasma (TABLE 7), nor tissue extracts (TABLES 4, 5, and 6) from normal or resistant animals produced noticeable toxic effects. Injections of tissues obtained from animals immediately after exposure to trauma were equally ineffective in producing deleterious changes. Extracts of spleen and of plasma were tested periodically for such activity by injecting rats intravenously via the jugular vein with two 2 ml doses 24 hours apart; no ill effects were observed. Toby and Noble[18] were not able to induce any toxic reactions with serum obtained immediately after trauma or from fully resistant rats when given to normal rats subcutaneously, intraperitoneally, or intravenously 1 or 48 hours before or immediately after trauma. If the lethal consequences of shock are caused by toxic factors resulting from tissue damage,[14,18,40–42] such noxious materials should be present in traumatized tissues.

Nature of Protective Extracts

The protective factor is water soluble and resistant to *p*H 5.5 and to boiling for at least 10 min. It is stable at 4° C for at least 1 week and at −20° C for several weeks. Preliminary studies indicate that it is nondialyzable and can be lyophilized. Boiled filtrates of plasma and spleen contain approximately 1 to 3 mg protein/ml.

It is now recognized that many carbohydrate-rich protein components exist in serum and tissues and appear to be involved in the response to stress. Thus a rise in protein-bound polysaccharides in the α_1-globulin fraction of serum was observed in a variety of diseases,[43] anemia,[3,33] and surgical trauma.[41] It appears likely that the active principle from trauma-resistant animals may be similar to these glycoproteins (mucopolysaccharide protein complexes) or perhaps to endotoxinlike components of mammalian tissues (lipopolysaccharide protein complexes).[45] Under conditions of stress these components either may be released more quickly from the cellular system or they may represent new products that are not normally present.

Other factors from reticuloendothelial organs have been reported. Ungar[46] claims to have isolated a hormonal substance, Splenine A, in crystalline form from splenic tissue; this substance is secreted by the spleen under the influence of the hypophysis and adrenal cortex. It occurs only in the spleen and blood, augments capillary resistance and decreases capillary permeability and bleeding time. It is believed to play a part in the control of protein metabolism and its adjustments to conditions of stress. However, it is rapidly hydrolyzed

Removal of the Spleen

Because of the striking protection afforded normal animals by the injection of spleen extracts from trauma resistant rats the susceptibility of animals after the removal of the spleen was investigated A group of normal adult female rats of the CF Nelson strain similar to those used in the replacement studies above, were splenectomized under light anesthesia and were exposed to 650 rotations in the Noble Collip drum 24 hours after operation Control animals underwent the same surgical procedures except that their spleens were left intact In the first experiment no significant changes in survival were noted (TABLE 8 group A) In fact all of the splenectomized animals survived while only 78 per cent of the controls lived Two days later the survivors were subjected to 900 turns all of the controls survived whereas only 56 per cent of the splenectomized animals lived These results suggested that a

TABLE 8
SUSCEPTIBILITY OF RATS TO DRUM TRAUMA AFTER SPLENECTOMY

Treatment	Revolutions	Day*	Survival
Group A			
Splenectomized	650	1	9/9 (100%)
Sham operated	650	1	7/9 (78%)
Splenectomized	900	3†	5/9 (56%)
Sham operated	900	3	7/7 (100%)
Group B			
Splenectomized	725	3	24/54 (44%)
Sham operated	725	3	6/18 (33%)

* Days following removal of the spleen
† Same animals exposed to 900 revolutions

lapse of 3 days is required for the effects of spleen deficiency to become apparent Subsequent experiments in which splenectomized rats without prior exposure to trauma were tumbled at 725 revolutions 3 days after splenectomy (TABLE 8 group B) however failed to show any significant difference between the control and splenectomized groups The survival of these animals in the various experiments ranged from 19 per cent to 67 per cent showing an over all average of 44 per cent for the splenectomized rats as compared to the control level of 33 per cent

The normal spleen therefore may not be concerned with the protection against drum trauma this would parallel the failure of normal spleen extracts to show any protective activity (TABLE 6) but since the rat is notorious in replacing spleen function by extramedullary tissues a true spleen deficiency at least in regard to a specific protecting principle may be unattainable

In this regard the average value of 44 per cent shown in TABLE 8 is really masking variations in survival that may be more informative in that it may be a reflection of the degree of replacement of splenic function by other tissues

but it has not been shown to induce resistance to lethal doses of endotoxin Fine *et al* reported that substantial quantities of lipopolysaccharide were extracted from shock plasma whereas no visible precipitate occurred in the corresponding fraction of normal plasma

On the other hand the state of irreversible hemorrhagic shock has been induced in the absence of bacterial factors in the germ free rat Since circulatory collapse seems to be associated with the same basic elements in both germ free and normal control animals, Zweifach *et al*[59] have suggested that the contribution of bacterial contamination to irreversibility is at best secondary in nature The demonstration of endotoxinlike substances in mammalian tissues that are capable of producing many phenomena previously ascribed only to bacterial endotoxins may explain some of the events of hemorrhagic shock and other conditions leading to vascular collapse[45]

In the present studies several acidified boiled plasma fractions were assayed for lipopolysaccharides according to the phenol water extraction method of Westphal[60] to determine whether endotoxins might be involved in the transfer of resistance Unlike the situation reported in hemorrhagic shock[10] however, no lipopolysaccharides were found in extracts of plasma from either normal or trauma resistant rats Along with the failure of these extracts to elicit toxic effects as previously discussed and the observation that bacterial endotoxins in the presence of serum are inactivated by heating[61] these data suggest that the protective capacity of the acidified boiled filtrates is not caused by the presence of bacterial endotoxins and that the symptoms of traumatic shock are probably due to some other perhaps common physiological mechanism

The relation of drum trauma and X irradiation is also presently under investigation It was found that conditioning animals to traumatic shock did not alter the survival of these animals significantly against lethal doses of X irradiation under the conditions of these experiments (McKenna and Zweifach[6], Reichard unpublished results) However preliminary studies have indicated that the administration of acidified boiled extracts of spleens from trauma resistant animals over a 2 day span may protect the normal animal against otherwise lethal doses of radiation These studies are being extended

It seems clear that drum trauma hemorrhage endotoxin poisoning and perhaps other stresses such as X irradiation may evoke common homeostatic readjustments that are fortified by procedures such as prior exposure to sublethal episodes of stress so that a steady state is maintained in spite of the normally lethal stimulus When these adjustments fail however similar manifestations are evident irrespective of the original stimulus and the organism lapses into circulatory failure The RES appears to play a central role in these compensatory responses since depressed RES function increases the susceptibility and eliminates induced resistance to these stresses whereas resistance to normally lethal doses is displayed following augmentation of RES function[8 10 11] Moreover in the present work the RES has been shown to be concerned with the production of an active principle present in the trauma resistant rat that is capable of protecting the normal animal against lethal consequences of drum trauma

full splenic function and produce sufficient protective material to compensate for the loss of spleen, whereas the resistance established after a single exposure of 650 turns may not be as complete and is more easily overcome or prevented

This situation is similar to that observed with Ferritin which was found to lower the survival of normal animals and overcome resistance once established but it had no effect on the development of resistance in normal animals[3]

Adrenalectomy

The role of the adrenal cortex in traumatic shock was investigated by subjecting adrenalectomized animals to 650 rotations in the tumbling apparatus 48 hours after operation (TABLE 11) Approximately 70 per cent of the control survived whereas all of the adrenalectomized animals succumbed The survival of animals receiving cortisone for 2 days prior and 2 days subsequent to adrenal removal was returned to normal however the administration of cortisone to normal rats failed to alter their resistance to drum trauma Noble and Collip[4] also observed that adrenalectomized rats were more susceptible than normals to drum trauma but they also found that pretreatment with cortin not only restored the resistance to control levels in adrenalectomized animals but raised the resistance of normal animals The failure of cortisone to affect the survival of normal animals has been confirmed by other investigations[63,64] On the other hand very large doses of cortisone that depress RES function[65,66] have been shown to increase the susceptibility of rats to trauma[3]

TABLE 11
RESISTANCE TO DRUM TRAUMA FOLLOWING ADRENALECTOMY AND CORTISONE TREATMENT

Treatment	Number of rats	Survival
Controls	18	12 (67%)
Controls + cortisone	18	13 (72%)
Adrenalectomy	18	0
Adrenalectomy + cortisone	18	11 (61%)
Adrenalectomy + physiological saline	18	1 (5%)

Rats received 650 turns in Noble Collip drum

Adrenalectomized animals also can be made resistant to drum trauma by prior exposure to sublethal episodes Toby and Noble[15] employed a training schedule that began with 50 turns and was increased every 2 days by 50 turns so that adrenalectomized rats eventually could tolerate 650 rotations In the present studies the following regime was used 50 100 and 150 turns on the first 3 days respectively followed by the routine training program of 4 days that is 200 turns on days 4 and 5 and 300 on days 7 and 8 On day 9 the animals were subjected to 725 rotations without mortality Another group of animals was exposed to the routine conditioning process of 4 days only but received 2 mg cortisone for 2 days prior to conditioning and 2 hours prior to each exposure With this treatment it was possible to subject adrenalectomized animals to the same training regime as normal animals for the most part preventing the effect of adrenal insufficiency

Further evidence that traumatic shock is accompanied by an adrenocortical

The occasional low survival of 19 per cent incorporated into this average value may represent slow or aberrant replacement and in reality may be the only true instance of splenic deficiency The high values of 67 per cent are also lost within the average of 44 per cent Like many other instances of homeostatic adjustment the tissues that assume the function of the spleen may over compensate by producing an excess of the protective principle

The effect of splenic deficiency on acquired resistance was also studied Rats made trauma resistant by the routine training program of 4 days and splen

TABLE 9

SURVIVAL OF TRAUMA RESISTANT RATS AFTER SPLENECTOMY

Revolutions	Days*	Survival
900	1	10/10 (100%)
900	5	10/10 (100%)
1100	15	8/10 (80%)
1300	17	8/8 (100%)
1500	20	7/8 (88%)

* Days following removal of the spleen from trauma resistant rats

TABLE 10

EFFECT OF SPLENECTOMY ON CONDITIONING TO DRUM TRAUMA

Day	Revolutions*	Mortality†	
		Splenectomized	Controls
1–2	200	0/9	0/9
3–4	300	0/9	0/9
5	900	0/9	0/9
7	1100	0/9	0/9
9	1300	1/8	2/9
12	1500	2/7	1/7
15	1700	0/6	0/6
18	2000	1/5	1/5

* Rats subjected to training regime on day 3 following removal of the spleen and sham operations

† No of rats succumbed/total no of rats tested

ectomized on the fifth day were tumbled at 900 turns 24 hours after operation No loss of resistance was observed (TABLE 9) These same animals were retested at intervals during the following 20 days at increasing levels of trauma only one of them succumbing from shock Thus the presence of the spleen does not seem to be crucial after resistance has been established

The fact that all sham operated rats in group A (TABLE 8) survived 900 rotations after prior exposure to 650 rotations whereas only 56 per cent of the splenectomized rats lived suggests that the splenectomized animal may have lost the ability to become trauma adapted Nevertheless the presence of the spleen was not essential for establishing tolerance since the operated rats were rendered trauma resistant by the routine conditioning procedure (TABLE 10) Again this gradual training regime may allow extramedullary tissues to assume

its ability to provide protection against other stresses such as infection bacterial endotoxins and X irradiation

References

1 Good R A & L Thomas 1952 Studies on the generalized Shwartzman reaction II The production of bilateral cortical necrosis of the kidneys by a single injection of bacterial toxin in rabbits previously treated with thorotrast or trypan blue J Exptl Med 96 625-641
2 Smith R T J Thomas & R A Good 1953 Generalized Shwartzman Reaction V Intravenous injection of colloidal iron or carbon on response of rabbits to meningococcal toxin Proc Soc Exptl Biol Med 82 712-715
3 McKenna J M & B W Zweifach 1956 Reticulo endothelial system in relation to drum shock Am J Physiol 187 263-268
4 Zweifach B W & L Thomas 1957 Influence of reticulo-endothelial system on response to hemorrhage and trauma AFS Bull 3 31 36
5 Zweifach B W B Benacerraf & L Thomas 1957 The relationship between the vascular manifestations of shock produced by endotoxin trauma and hemorrhage II The possible role of the reticulo endothelial system in resistance to each type of shock J Exptl Med 106 403 414
6 Beeson P B 1947 Tolerance to bacterial pyrogens II Role of the reticulo endothelial system J Exptl Med 86 39-44
7 Beeson P B 1947 Effect of reticulo endothelial blockade on immunity to the Shwartzman phenomenon Proc Soc Exptl Biol Med 64 146-149
8 Windle W F W W Chambers W A Ricker L G Ginger & H Koenig 1950 Reaction of tissues to administration of a pyrogenic preparation from a Pseudomonas species Am J Med Sci 219 422-426
9 Fine J 1956 The effect of peripheral vascular collapse on the antibacterial defense mechanisms Ann N Y Acad Sci 66(2) 329 336
10 Fine J E D Frank H A Ravin S H Rutenberg & F B Schweinburg 1959 The bacterial factor in traumatic shock New Engl J Med 260 214-220
11 Fine J S Rutenberg & F B Schweinberg 1959 The role of the reticuloendothelial system in hemorrhagic shock J Exptl Med 110 547 560
12 Reichard S M A S Gordon & C F Tessmer 1960 Humoral mechanisms in protection against traumatic shock role of the RES *In* Reticuloendothelial Structure and Function J H Heller Ed Ronald Press New York N Y
13 Division of Veterinary Medicine 1957 Establishment and maintenance of a disease free colony Walter Reed Army Institute of Research U S Govt Printing Office 0-423831
14 Noble R L & J B Collip 1942 A quantitative method for the production of experimental traumatic shock without hemorrhage in unanesthetized animals Quart J Exptl Physiol 31 187-199
15 Biozzi G B Benacerraf & B N Halpern 1953 Quantitative study of the granulopectic activity of the reticulo endothelial system II A study of the kinetics of the granulopectic activity of the R E S in relation to the dose of carbon injected Relationship between the weight of the organs and their activity Brit J Exptl Pathol 34 441-457
16 Hyman C & R L Paldino 1954 Influence of reticulo endothelial blockade and stimulation on the rate of disappearance of Evans blue from the circulation Am J Physiol 179 594-598
17 Reichard S M & A S Gordon 1956 Adrenal influences on distribution of injected colloidal thorium Am J Physiol 186 63-66
18 Toby C G & R L Noble 1944 Factors affecting the development of resistance to traumatic shock in the rat Can J Research 22(Sect E) 9 88
19 Zahl P A S H Hutner & F S Cooper 1943 Noble Collip Shock Therapeutic effects with autonomic depressants motion factors J Pharmacol Exptl Therap 77 143 150
20 Zweifach B W & L Thomas 1957 The relationship between the vascular manifestations of shock produced by endotoxin trauma and hemorrhage I Certain similarities between the reactions in normal and endotoxin tolerant rats J Exptl Med 106 385-401
21 Griswold R L & I Gray 1957 Conditioning of rats to tumbling trauma by electroconvulsive shock Am J Physiol 189 504 508
22 Chambers R B W Zweifach & B E Lowenstein 1943 Circulatory reactions of rats traumatized in the Noble Collip drum Am J Physiol 139 123 128

response was shown by McLaughlin and Gray,[67] who found a rise in plasma concentration of corticosterone and 17 hydroxycorticosterone after drumming Levels immediately after trauma increased proportionally to the number of revolutions but returned to normal within 24 hours Levels after conditioning were raised to the same extent but fell to normal within 2 to 3 hours (J Mc Laughlin, Jr, personal communication) It seems clear that small amounts of adrenal steroids (maximum about 50 µg /100 ml plasma) could not account for the protective action of plasma extracts, preparations were also made 24 hours after the last drumming a time when levels had returned to normal

These observations support the concept that the adrenal cortex plays a permissive role in stress [68, 69] Adrenal cortical hormones are necessary for the response to stress to occur, and their sustained secretion is required to main tain the reaction, but the adrenal hormones are not directly responsible for the typical stress reactions In some way, they permit the tissues to respond to the stressful stimulus Since the adrenal cortex[70, 71] and the RES[12, 7] are both involved in the response to stresses of various types such as trauma cold heat, toxins, radiation and infections the reaction of the RES in stress may be mediated through, or influenced by, the hypophyseal adrenal channel In this regard it was previously demonstrated that the phagocytic activity of the RES is influenced by the secretions of the anterior pituitary and adrenal cortex [73, 74] Thus the RES in the presence of adrenal hormones may be re sponsible for many of the physiological phenomena associated with stress This suggestion is in accordance with the present demonstration that extracts of spleen and plasma are capable of altering the physiological adaptation to trauma possibly through a modification of RES function

Summary

Impairing the function of the RES was observed to increase the susceptibility of rats to traumatic shock and to eliminate acquired resistance produced in animals through repeated exposures to sublethal doses of shock Thus it was considered that the RES might be responsible for the protection of trauma resistant rats against otherwise lethal insults of trauma (Noble Collip drum) Acidified boiled filtrates prepared from plasma and spleens of trauma resistant rats effectively increased survival of normal unconditioned rats exposed to trauma These plasma and spleen filtrates were administered subcutaneously in four doses over a two day period prior to exposure of the recipient rats to lethal drum trauma Similar preparations of the liver were ineffective as were tissues and plasma obtained from normal unconditioned rats The RES therefore seems to be concerned in the production or concentration of a humoral factor evoked during drum trauma which affords protection against this stress The active principle is capable of altering the physiological adaptation to trauma possibly through modifying RES function The adrenal cortex appears to play a supportive role, making it possible for the RES to function in the homeo static adjustments necessary to overcome ultimate circulatory failure in trau matic shock

Studies are being extended to purify and characterize this humoral factor produced in the traumatized rat and to determine its time of appearance and

52 FREEDMAN H H 1960 RES and passive transfer of endotoxin tolerance Ann N Y Acad Sci 88(1) 99
53 BENACERRAF B M M SEBESTYEN & S SCHLOSSMAN 1959 A quantitative study of the kinetics of blood clearance of P[32] labelled Eschericia coli and Staphylococci by the reticuloendothelial system J Exptl Med 110 27-48
54 FINE J 1957 The bacterial factor in experimental shock In Shock and Circulatory Homeostasis 37 102 H D Green Ed Josiah Macy Jr Foundation New York N Y
55 PILLEMER L 1956 The nature of the properdin system and its interactions with polysaccharide complexes Ann N Y Acad Sci 66 2) 233 246
56 PILLEMER L L BLUM I H LEPOW O A ROSS E W TODD & A C WARDLAW 1954 The properdin system of immunity I Demonstration and isolation of a new serum protein properdin and its role in immune phenomena Science 120 279 285
57 SMIDDY F G & J FINE 1957 Host resistance to hemorrhagic shock X Induction of resistance by shock plasma and by endotoxin Proc Soc Exptl Biol Med 96 558 562
58 SHORR E S BAEZ D B METZ & B W ZWEIFACH 1952 Cross resistance to hemorrhagic shock in rats made resistant to drum trauma relation to hepatic VDM inactivation mechanisms Federation Proc 11 147
59 ZWEIFACH B W H A GORDON M WAGNER & J A REYNIERS 1958 Irreversible hemorrhagic shock in germfree rats J Exptl Med 107 437-450
60 WESTPHAL O O LUDERITZ & F BISTER Uber die Extraktion von Bakterien mit Phenol Wasser Z Naturforsch 7b 148-155
61 PETERSDORF R G & I L BENNETT JR 1957 Studies on the pathogenesis of fever VI The effect of heat on endogenous and exogenous pyrogen in the serum of dogs Bull Johns Hopkins Hosp 100 197 08
62 MCKENNA J M & B W ZWEIFACH 195 Comparative study of the physiological effects of x ray radiation and resistance to traumatic shock Radiation Research 6 126-136
63 NORTH W C & J A WELLS 1951 Protective effect of certain drugs against shock induced by the Noble Collip drum J Pharmacol Exptl Therap 103 356
64 FRANK H A S JACOB H A F WEIZEL, L REINER R COHEN & J FINE 1955 Effects of ACTH and cortisone in experimental hemorrhagic shock Am J Physiol 180 282 286
65 THOMAS L 1953 Cortisone and infection Ann N Y Acad Sci 56(4) 99-811
66 HELLER J H 1955 Cortisone and phagocytosis Endocrinology 56 80-85
67 MCLAUGHLIN J JR & I GRAY 1956 Biochemical response to trauma II Corticosterone and 17 hydroxycorticosterone levels in plasma of rats subjected to tumbling trauma Research Report WRAIR 86-56
68 INGLE D J E O WARD & M H KUIZENGA 1947 The relationship of the adrenal glands to changes in urinary non protein nitrogen following multiple fractures in the force fed rat Am J Physiol 149 510-515
69 ENGLE F L 1952 Metabolic aspects of hemorrhagic and traumatic shock In Shock and Circulatory Homeostasis 26-161 H D Green Ed Josiah Macy Jr Foundation New York N Y
70 SAYERS G 1950 The adrenal cortex and homeostasis Physiol Revs 30 241 320
71 SELYE H 1950 The Physiology and Pathology of Exposure to Stress Acta Inc Medical Publ Montreal Canada
72 BERRY L J & T D SPIES 1949 Phagocytosis Medicine 28 239-300
73 REICHARD S M A EDELMANN & A S GORDON 1956 Endocrine influences upon the uptake of colloidal thorium J Lab Clin Med 48 431-441
74 REICHARD S M A EDELMANN & A S GORDON 1956 Adrenal and hypophyseal influences upon the uptake of radioactive gold (Au[198]) by the reticulo-endothelial system Endocrinology 59 55-68

23 CLARKE A P W & R A CLEGHORN 1942 Chemical study of tissue changes in adrenal insufficiency and traumatic shock Endocrinology 31 597 606
24 GRAY I W J JORDAN N D GOLDBERG & W D CASH 1957 Mechanism of the protective action of magnesium in mechanical trauma involving epinephrine and nor epinephrine Am J Physiol 189 229-232
25 NEUFELD A H C G TOBY & R L NOBLE 1943 Biochemical findings in normal and trauma resistant rats following trauma Proc Soc Exptl Biol Med 54 249 252
26 MCSHAN W H V R POTTER A GOLDMAN L G SHIPLEY & R K MEYER 1945 Biological energy transformations during shock as shown by blood chemistry Am J Physiol 145 93-106
27 LEPAGE G A 1946 Biological energy transformations during shock as shown by tissue analyses Am J Physiol 146 267-281
28 LACKER L M MICHAELIS & W R MARTIN 1958 Effect of shock on rat heart and brain mitochondria Proc Soc Exptl Biol Med 98 164-167
29 NOBLE R L 1942 The development of resistance by rats and guinea pigs to amounts of trauma usually fatal Am J Physiol 138 346-351
30 GORDON A S S J PILIERO W KLEINBERG & H H FREEDMAN 1954 A plasma extract with erythropoietic activity Proc Soc Exptl Biol Med 86 255-258
31 GORDON A S S J PILIERO P T MEDICI C D SIEGEL & M TANNENBAUM 1956 Attempts to identify site of production of circulating erythropoietin Proc Soc Exptl Biol Med 92 598 602
32 BORSOOK H 1959 A discussion of humoral erythropoietic factors Ann N Y Acad Sci 77(3) 725-736
33 RAMBACH W A J A D COOPER & H L ALT 1959 The nature of erythropoietin Ann N Y Acad Sci 77(3) 623-6 6
34 FARR R S S L CLARK JR J F PROFFITT & D H CAMPBELL 1954 Some humoral aspects of the development of tolerance to bacterial pyrogens in rabbits Am J Physiol 177 269-278
35 UNGAR G 1943 Experimental traumatic shock Factors affecting mortality and effect of therapeutic agents (ascorbic acid and nupercaine) Lancet 244 421-424
36 LANDY M R C SKARNES F S ROSEN R J TRAPANI & M J SHEAR 1957 Inactivation of biologically active (endotoxic) polysaccharides by fresh human serum Proc Soc Exptl Biol Med 96 744-747
37 MILLICAN R C H Tabor & S M ROSENTHAL 1952 Traumatic shock in mice Comparison of survival rates following therapy Am J Physiol 170 179 186
38 ROSENTHAL S M & R C MILLICAN 1954 The role of fluids electrolytes and plasma proteins in experimental traumatic shock and hemorrhage Pharmacol Rev 6 489 520
39 GRAY I W W TOLSON N A CARROLL & R MUNDY 1957 Effect of subcutaneous fluids with hyaluronidase on mortality in laboratory animals subjected to hemorrhagic and mechanical trauma J Appl Physiol 10 284 288
40 CANNON W B 1923 Traumatic Shock Appleton New York N Y
41 MOON V H 1938 Shock and Related Capillary Phenomena Oxford Univ Press New York N Y
42 NOBLE R L & J B COLLIP 1942 Adrenal and other factors affecting experimental traumatic shock in the rat Quart J Exptl Physiol 31 201 210
43 WINZLER R J 1958 Glycoproteins of plasma In Chemistry and Biology of Mucopolysaccharides 245 267 G E W Wolstenholme & M O Conner Eds Little Brown Boston Mass
44 SCHUMACHER G 1956 Klinische und experimentelle Untersuchungen uber Verhalten und Funktion der Alpha 2 Globuline Verhandl deut & Ges inn Med 62 347 353
45 LANDY M & M J SHEAR 19 7 Similarity of host responses elicited by polysaccharides of animal and plant origin and by bacterial endotoxins J Exptl Med 106 77 97
46 UNGAR G 1945 Endocrine function of the spleen and its participation in the pituitary adrenal response to stress Endocrinology 37 329-340
47 UNGAR G 1947 Etudes biochimiques et physiologiques sur deux substances actives produites par la rate J physiol Paris 39 219 248
48 TORO I 1948 Die humorale Regulierung der Speicherung in der Leber Acta Anat 5 311 347
49 POMERAT C M & M F ORR 1949 Further observations on a macrophage promoting factor (MPF) in the blood serum Anat Record 103 496
50 TULLIS J L & D M SURGENOR 1956 Phagocytosis promoting factor of plasma and serum Ann N Y Acad Sci 66(2) 386-390
51 ROWLEY D 1958 Bactericidal activity of macrophages in vitro against Escherichia coli Nature 181 1738 1739

similar rates during the first few hours following injections significant differences can be demonstrated if the removal of these proteins is followed for days or weeks It is therefore very difficult to evaluate any of these turnover studies without specific knowledge of the label and its behavior in the particular system studied

Our studies on the role of the RES in the movement of protein from the

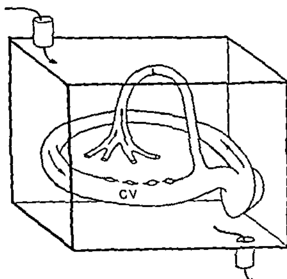


FIGURE 1 Sch mat c diagram of the three circulations in which proteins are involved Th precursors of the plasma proteins enter the body (*large box*) where proteins are synthesized and ultimately broken down and their products eliminated (*lower arrow*) Plasma prot r c rculate w th n the card ovascular system (CV) where a fraction of the total number of molecules lea es the several capillary beds by a mechan sm still unknown the molecules may pass between th e ndothelial cells in some passive way or may be tran ported acti ely by some ll mecha us n Once n tissue flu d the prote n molecules are picked up by the lymphatics (L) and returned to the ca diovascular system

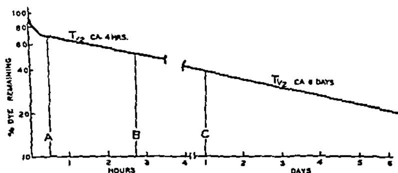


FIGURE 2 T me course of disappea ance of labeled protein from the circulation To A is the period of mixing to B is the per od before lymphatic return of label and beyond C the rate is the measure of m tabol c breakdown

POSSIBLE ROLE OF THE RETICULOENDOTHELIAL SYSTEM IN PROTEIN TRANSPORT*

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An individual plasma protein molecule in a living organism may be considered to be simultaneously in three distinct circulations (FIGURE 1) First it is obviously circulating within the continuous endothelial tubes that constitute the cardiovascular system second the proteins of the plasma move in a circuit from the blood to and through the tissue fluid and back to the circulation via the lymphatics, and third the protein molecule is in a metabolic cycle that starts with the uptake of its precursors from the environment and ends with the elimination of breakdown products through the kidneys The possible role of the reticuloendothelial system (RES) in the metabolic breakdown of the plasma proteins has been explored by several workers[1] It has also been suggested that the RES might play a distinct role in the movement of protein from the vascular to the extravascular compartment [4]

The rate of movement of the protein molecules in each of these several circuits may be estimated by experimental methods The movement around the cardiovascular system is measured roughly by the circulation time To distinguish the rate at which proteins move through the blood tissue fluid lymphatic circuit from the rate at which they are metabolized requires analysis of kinetics of removal of labeled protein from the circulation The concentration of marked molecules injected into the circulation falls at a rapid non exponential rate during the early mixing period then at a somewhat slower simple exponential rate characterizing the escape of the label from the vascular compartment This rate remains constant and is measured easily for about 2 hours At the end of this time the return of labeled protein via the lymphatics is about equal to the rate of disappearance of label from the circulation and a new slower exponential removal rate is established reflecting the metabolic destruction of the labeled protein[5] (FIGURE 2)

In anesthetized rabbits the rate of disappearance between 30 and 100 min after injection of Evans blue[2] was found to be an acceptable index of the escape of protein from the circulation for the labeled molecules escaping from the circulation are replaced by largely unlabeled protein molecules from the lymph

Studies of exchange or metabolic breakdown of proteins have generally made use of variously labeled protein molecules More careful measurements have focused attention on the possible difference between labeled and native protein molecules The information obtained from many comparisons of different forms of protein labeling strongly suggests that various labels show various degrees of adequacy for studying each of these several phenomena Partially denatured proteins are removed from the circulation[6] or are ultimately metabolized[7] more rapidly than are carefully labeled allegedly undenatured proteins Furthermore in a recent paper[8] it has been pointed out that although iodine labeled proteins and Evans blue labeled proteins are cleared from the blood at

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University of Southern California colony from the University of California colony at Berkeley and from the colony at Lobund Institute University of Notre Dame Notre Dame Ind give substantially the same results (FIGURE 3)

TABLE 2

DISAPPEARANCE OF T 1824 FROM THE CIRCULATION OF NORMAL AND GERM FREE RATS (LOBUND COLONY)

Con t l				C m f			
Co t l /m		Th t t mn		C t l /m		Th t t /m	
C1	0 223	C4	0 100	A15	0 365	A16	0 175
C3	0 223	C6	0 108	A17	0 346	A14	0 169
C5	0 238	C8	0 107	A13	0 331	A12	0 161
C7	0 223	C10	0 104	A11	0 346		
Mean	0 227		0 103		0 347		0 168
Sigma	±0 004		±0 004		±0 013		±0 —

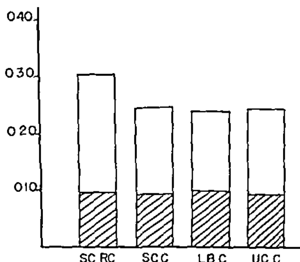


FIGURE 3 Comparison of rates of disappearance of labeled proteins from circulation of rabbits (SC RC) and rats of the University of Southern California strain (SC C) the Lobund strain (LB C) and the University of California strain (UC C) In each case the total height of the column indicates the mean of the disappearance rates (in per cent per minute) from circulation measured in a group of normal animals Height of shaded portion indicates disappearance measured after pretreatment with Thorotrast

The height of each column represents the average removal rate from the group of untreated rats and the shaded portion of the colum represents the average removal rate from the Thorotrast treated animals

An opportunity to check on the second objection to our early experiments was afforded us by Harold Tarver of the Department of Biochemistry at the

vascular to the extravascular compartment started with our findings in a series of studies where we measured the rate of removal of Evans blue from the circulation of rabbits.[9-11] We found that several procedures that increased the activity of the RES also increased the rate of removal of labeled protein from the circulation; on the other hand, blocking the RES considerably delayed the removal of the labeled protein. These studies gave consistent results with a variety of stimulant and blocking agents. However, all of these data were obtained on anesthetized rabbits of a uniform stock, and extrapolation to other forms required caution. Another argument against the validity of these measurements was the possibility that the Evans blue was not truly a label for the native protein and that we were in fact determining the rate at which a foreign agent, namely, the dyed protein, was being removed by the RES.

To satisfy the first objection, we modified our technique to measure protein disappearance from the circulation of rats. Animals weighing about 150 gm

TABLE 1
RATES OF DISAPPEARANCE OF LABELED PROTEIN IN RATS

	Normal				Thorotrast pretreated		
	T 1824* %/min	S %/min	S/Dy		T 1824 %/min	S %/min	S/Dy
A	0.248	0.244	0.985	C	0.080	—	—
B	0.234	0.207	0.886	D	0.0735	0.0885	1.2
				I	0.0960	0.0943	0.981
E	0.245	0.241	0.983				
F	0.241	0.230	0.956	G	0.0907	0.0846	0.934
				H	0.0933	0.0961	1.03
Mean	0.242	0.231	0.952		0.0867	0.0909	1.04
Sigma	±0.009	±0.016	±0.046		±0.0095	±0.0045	±0.082

* Evans blue

were anesthetized with sodium pentobarbital given intraperitoneally. Appropriate quantities of dye were injected into the exposed femoral vein, and the animals were prepared for arterial sampling from the abdominal aorta. Precautions were taken to prevent cooling and dehydration of the viscera. At 20, 40, 60, 80, and 100 min after injection of the dye, 1 ml samples were taken from the aorta, using 26-gage needles and a heparin-rinsed syringe. Samples were transferred to Wintrobe hematocrit tubes, centrifuged, and then treated as in the case of our earlier studies on the rabbits.[10] This method of sampling proved to be satisfactory. In several series of rats we established the fact that the rate at which Evans blue was removed from the circulation between 30 and 100 min was consistent and easily measured. The rate averaged about 0.23 per cent/min and was diminished to about 0.1 per cent/min by pretreatment with colloidal thorium dioxide (Thorotrast).* The depression obtained with Thorotrast pretreatment in the rat was about the same as that observed in the rabbit (TABLES 1 and 2). It is interesting to note that rats from the

* Obtained from Testagar & Co., Inc., Detroit, Mich.

fortuitously equally foreign this experiment would support the idea that the removal rates of Evans blue during the period that we are using is probably an adequate measure of the movement of native protein certainly, it is an adequate substitute for the S^{35} labeled protein We therefore feel more secure in supporting our original proposition that the RES is important in the transfer of protein from the vascular to the extravascular compartment[8]

Germ free animals were reported to have a morphologically underdeveloped RES[11] and the protective functions of this system were minimum It seemed possible that in these animal the RES might still be involved in protein trans

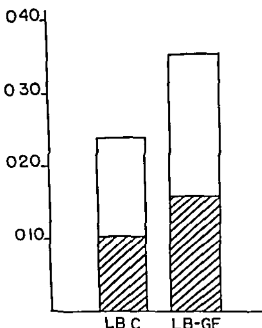


FIGURE 5 Comparisons of Evans blue disappearance rates in conventional and germ free rats (*See* caption to FIGURE 3)

port To check this possibility the following experiments were performed Helmut Gordon made available to us a group of germ free and conventional rats from the Lobund colonies for acute experiments Evans blue removal rates were determined *in germ free animals immediately after they were taken* from their rearing chambers The dye for injection was sterilized and all precautions against contamination were taken Evans blue is removed from the circulation of germ free rats more rapidly than from controls or from conventional rats of the Lobund colony However in both the germ free and the conventional animal RES blockade decreased the rate of removal of the labeled protein from the circulation by almost an equivalent percentage All of the data are given in TABLE 2 and FIGURE 5 Why the removal of protein from the circulation of the germ free animal was more rapid than the control rate is

University of California Not only did he provide us with a supply of purified rat plasma proteins labeled internally with S^{35} but he generously placed at our disposal the laboratory facilities and the animals for all the necessary determinations in his laboratory The proteins used were prepared by the method of Ulrich *et al*[1] and probably contained less than 5 per cent labeled globulin and were most likely not at all denatured

The removal of the 2 types of labeled protein from the circulation was measured simultaneously in individual rats First Evans blue labeled rat albumin

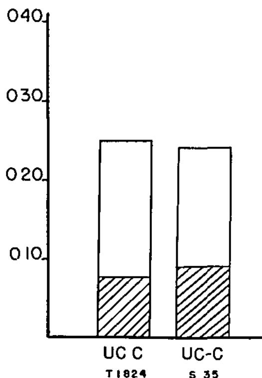


FIGURE 4 Comparison of removal rates for Evans blue and S^{35} labeled proteins (*See* caption to FIGURE 3)

and then, to minimize the possibility of double labeling S^{35} labeled rat albumin were injected from separate syringes at intervals thereafter arterial plasma samples were analyzed for both the dye and the sulfur label The rates of removal of both compounds were almost identical the ratio of the removal rates of any individual animal deviated by less than 5 per cent from unity Pretreatment with Thorotrast had an equal effect on the transfer of the S^{35} and Evans blue labeled protein The data given in TABLE 1 are summarized in FIGURE 4 There is no difference in the rate at which the 2 labels are removed from the circulation of rats nor is there any obvious difference between the effect of Thorotrast on the removal rates of either the Evans blue or the S^{35} labeled protein

Unless the sulfur labeled albumin and the Evans blue labeled protein were

half time approaching one hour It is not impossible that the protein molecule should be removed by a closely related process with the half times we have found

It is our basic argument that under normal conditions native proteins are transported from the vascular to the extravascular compartment to a large extent as a result of the activity of the macrophages of the RES

Acknowledgments

We are indebted to Harold Tarver for his courtesy and assistance We also thank Warner Chilcott Laboratories Division of the Warner Lambert Pharmaceutical Company Morris Plains N J for the generous supplies of Evans blue dye with which they supplied us

References

1 Freeman T & H Gordon & J H Humphrey 1958 Distinction between catabolism of native and denatured protein by the isolated liver after carbon loading Brit J Exptl Path 1 39 452
2 Jancsó M 1947 Histamine as a physiological activator of the reticuloendothelial system Nature 160 227
3 Hyman C 1953 Role of the reticulo endothelial system in protein turnover Abstr 19th Intern Physiol Congr 486
4 Jancsó M & A Jancsó-Gabor 1954 Die Speicherung von Blutproteinen in den Histiozyten nach vorhergegangenen der Histaminein Wirkung Experientia 10 2 6
5 Wasserman K & H S Mayerson 1952 Dynamics of lymph and plasma protein exchange Cardiologia 21 296
6 Benacerraf B G Biozzi B N Halpern C Stiffel & D Mouton 1957 Phagocytosis of heat-denatured human serum albumin labeled with ¹³¹I Brit J Exptl Path 1 38 35
7 Goldsworthy P D & W Volwiler 1958 Comparative metabolic fate of chemically (I ¹³¹) and biosynthetically (C ¹⁴ or S) labeled proteins Ann N Y Acad Sci 70(1) 26
8 Garrow J S & J C Waterlow 1959 Evans blue dye as a tracer for human plasma albumin Clin Sci 18 35
9 Paldino R L M Sosnow & C Hyman 1953 Changes in protein bound dye disappearance rate as a result of evisceration and nephrectomy Circulation Research 1 242
10 Hyman C & R L Paldino 1954 Influence of reticulo endothelial blockade and stimulation on the rate of disappearance of Evans blue from the circulation Am J Physiol 179 594
11 Bernick S C Hyman & R L Paldino 1955 Histological studies of the influence of Thorotrast or the intrahepatic distribution of T 1824 Am J Physiol 182 232
12 Ulrich F C H Li & H Tarver 1954 Electrophoresis of rat plasma II Preparation of S labeled albumin Arch Biochem Biophys 50 471
13 Gordon H A 1955 Morphological and physiological characterization of germ free life Ann N Y Acad Sci 78(1) 208
14 Zweifach B W 1959 Vascular Endothelium in the Arterial Wall A I Lansing Ed Williams & Wilkins Baltimore Md
15 Jancsó M 1956 Speicherung Akademiai Kiádó Budapest Hungary
16 Moore D H & H Ruska 1957 The fine structure of capillaries and small arteries J Biophys Biochem Cytol 3 457
17 Dobson E L & H B Jones 1951 The behavior of intravenously injected particulate matter Acta Med Scand 144(Suppl 273)

not clear nevertheless, these data suggest that the pathways by which labeled protein leaves the circulation in the normal animal and the germ free animal are probably comparable, since they both are blocked to about the same extent by Thorotrast

If Thorotrast blockade implies an initial uptake of this colloid by the RES and subsequent interference with the uptake of other agents (in our case labeled protein) one must conclude that the RES cells of germ free animals are capable of picking up colloidal Thorotrast as well as labeled protein

Discussion

The data here presented indicate that (1) the movement of labeled protein from the vascular compartment of rats is depressed when the RES is blocked by Thorotrast (2) the movement of either Evans blue or the internally sulfur labeled protein is effected to about an equal degree and (3) that this movement of protein is at least qualitatively the same in germ free and normal rats

We stress the fact that our conclusions and our data refer to the particular circumstances of the experiments described we do not imply that these labeled proteins are metabolized at equal rates or that the RES handles them in a similar fashion except for the initial removal from the circulation

It is interesting to speculate on the fate of a protein molecule once it has been taken up into the macrophage It is possible that since the molecule is broken down at this point a new molecule must be resynthesized that contains the label to account for the fact that many investigators have shown the appearance of labeled proteins in the lymph soon after their administration into the circulation[6] or perhaps the protein molecules that appear in the lymph are those that have not passed through the RES In our case about one third of the protein removal still continues in spite of blockade of the RES however we have no measure of the completeness of the blockade Alternatively macrophages may be capable of egesting or passing intact protein molecules to the extravascular side In this concept the macrophage would act as a window or transfer system built into the vascular wall to provide means for the active transfer of the protein molecules from one side to the other Zweifach[14] cites the suggestion of Jancso[15] that certain perivascular phagocytes may have processes that extend between the endothelial cells to place them in direct contact with the circulating blood Such an arrangement would also serve to move materials from the blood to the extravascular space There is little evidence of any kind to help us decide among these several possibilities and clarification of the exact details of the mechanism awaits further studies

It is difficult at the present time to identify the mechanism by which the soluble proteins are taken into the macrophage It may be that some special phenomenon akin to pinocytosis[1] is involved or it may be that the protein molecules are carried along with the other agents that are being removed from the circulation by macrophages As we have pointed out before the relatively long half times and the small dimensions of the particles being considered are not of them elves disproof of phagocytosis Dobson and Jones[17] showed that the nonsedimentable small particle fraction of a mixed chromic phosphate colloid was removed from the circulation by phagocytosis with a

observed that over 90 per cent of the dose disappeared at an exponential rate The greater the load of chylomicrons the slower the fractional rate of disappearance[12,18] French and Morris[19] found this to be true also in rat This again is the same dynamics as observed with particles known to be phagocytized

In the rat rabbit and dog stainable fat can be demonstrated in phagocytic cells of the liver after a fatty meal and an increase in liver lipid is shown by analysis This increase in liver fat does not take place if large amounts of colloid are injected during the time the fed fat is being transported as chylomicrons[9,25]

The extent to which the RE system ordinarily takes up fat as just described is controversial It seems clear however that adipose tissue does have the ability to remove TG fat from the blood[26-28] In this connection Clement[29] has called attention to adipose tissue as originating in a primary adipose tissue with a reticuloendothelial structure

The accumulation of lipid from labeled chylomicrons has been observed *in vitro* with the L strain cells of mouse fibroblasts[30] and also with the MB III strain[31] these cells later metabolized some of this lipid during their growth[30]

When two different colloids are simultaneously injected the more avidly phagocytized of them is removed from the blood almost completely before clearance of the less avidly phagocytized particle is begun[17] If the less avidly phagocytized particle is injected first its phagocytosis is suspended during removal of the second type of particle and resumed afterward The dynamics of this process have been shown by Neveu *et al*[32] to apply to the removal of cholesterol-containing rabbit lipoproteins and chylomicrons from the blood of rats French and Morris[19] found that the disappearance curve of chylomicrons resembled very closely the disappearance of intravenously labeled inorganic colloid where variation in particle size accounts for the complexity of the disappearance curve This is because smaller particles account for slowly removed tail while larger particles of the same colloid are removed more rapidly French and Morris injected large and small chylomicrons separately[19] The large chylomicrons disappeared at a faster rate than the small ones This is easy to account for if the RES took up the particles but difficult to explain on other bases For instance intravascular hydrolysis or any other enzymatic process should act more rapidly on smaller particles because of the greater percentage of this molecules exposed on the surface Similarly if chylomicrons go through the wall of the liver sinusoids by entering the spaces or holes found therein by Fawcett[33] smaller particles should pass through more rapidly than larger ones since they are less likely to hit the boundaries of the hole Perhaps only large chylomicrons are phagocytized while smaller particles pass through the spaces of Fawcett in the sinusoidal epithelium and are metabolized by parenchymal cells

T H Spaet (personal communication) has suggested that the differential observed between the disappearance rate of large and small particles may reflect the rate of exit not of particles but simply of mass of material since there is a very much larger mass per particle (proportional to the cube of the radius) for large particles than there is for small ones

LIPIDS AND THE RETICULOENDOTHELIAL SYSTEM

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In this review I do not propose to prove anything but shall merely list a number of findings most of them very recent, which continue to make plausible the old concept of *functional connection between the reticuloendothelial* system (RES) and lipid metabolism

To begin with one of the most recent findings, I cite Di Luzio's analysis of Kupffer's cells,[1] characterized by an elevated cholesterol concentration in both free and ester fractions The two forms of cholesterol were present in the same ratio as elsewhere These proportions may be the result of passive uptake but they also may have occurred as the result of active metabolism for macrophages in the skin[2] peritoneal cavity[3] and sinus reticular cells of the lymph nodes[4] have been shown to be capable of esterifying cholesterol ingested from artificial suspension and those of the sternal lymph nodes to be also capable of hydrolyzing ingested ester cholesterol[4]

The capability of the RES to ingest lipid has long been dramatized in the lipidotic diseases or lipidoses a synonymous name for these diseases is reticuloendothelioses In particular in Hand Schuller Christian Niemann Pick Hurler's and Gaucher's diseases there is proliferation of histiocytes which become filled with cholesterol esters[5,6]

RE cells are probably capable of not only accumulating lipid but also of releasing it Lautsch *et al*[7] produced prompt transient hypercholesteremia in the normal rabbit by the injection of colloids If the injections were repeated atherosclerotic lipid deposits could be found in the aorta Similar findings have been published using guinea pigs,[8] in which colloid injection was observed to bring about a rise of both serum cholesterol and vitamin A esters Carotenes and vitamin A esters are fat soluble and collect in lipid deposits[9] Krinsky *et al*[10] have concluded that the reticuloendothelial system is the site for removal of vitamin A esters from plasma

Nearly all dietary lipid is delivered to the blood in a finely emulsified state in the thoracic duct chyle the small particles being called chylomicrons Intravascular hydrolysis accounts for the disappearance of only 5 per cent or less of this lipid[11,12] There is considerable evidence to show that the chylomicrons can disappear from the serum as a unit[13] The liver is capable of removing chylomicrons from the serum and continues this capability even as an isolated perfused organ[14,15] Brauer *et al*[16] have shown that the phagocytic activity of the liver RE is approximately the same in the isolated organ as *in vivo* As it happens however the dynamics of the removal of chylomicrons from serum are fairly similar to the principal characteristics of the phagocytosis by the RES of particles circulating in the blood Intravenous colloids known to be phagocytized by the RES disappear from the blood at an exponential rate[17] When Havel and Fredrickson[12] injected dogs with chylomicrons in which the triglycerides (TG) contained palmitate C^{14} they

24 Rice L I M C Schotz J I Powell & R B Alfin Slater 1954 Effect of reticulo en lothel al blocking agents on plasma and liver cholesterol levels in the rat Am J Physiol 178 483
25 Waddell W R R P Geyer E Clarke & F J Stare 1954 Function of the reticulo-endothelial system in the removal of emulsified fat from blood Am J Physiol 177 90
26 Bragdon J H & I S Gordon Jr 1958 Tissue distribution of C after the intra venous injection of label d chylomicrons and unesterified fatty acids in the rat J Clin Invest 37 574
27 Dole V P A T James P W Webb M A Rizack & M F Sturman 1959 The fatty acid patterns of plasma lipids during alimentary lipemia J Clin Invest 38 1544
28 Stein Y & B Shapiro 1959 Assimilation and dissimilation of fatty acids by rat liver Am J Physiol 196 1238
29 Clement G 1951 Mobilization and transport of reserve fats Presse med 59 1442 *See also* Vellios F J Baez & H Shumacker 1958 Lipoblastomatosis A tumor of fetal fat different from hibernoma Am J Pathol 34 1149
30 Bensch K C & D W King 1959 The accumulation of lipid in L strain cells Federation Proc 18 468
31 Bailey J M G O Gey & M G Gey 1959 Utilization of lipids by cultured mammalian cells Proc Soc Exptl Biol Med 100 686
32 Nevel T C Biozzi B Benacerraf C Stiffel & B N Halpern 1956 Role of reticulo endothelial system in blood clearance of cholesterol Am J Physiol 187 269
33 Fawcett D W 1955 Observations on the cytology and electron microscopy of hepatic cells J Natl Cancer Inst 15 1475

This review has been neither objective nor dispassionate Evidence contradictory to some of the conclusions given has been ignored However it would seem quite surprising if when at last everything about fat transport and metabolism is completely known the RES were found not to be involved in any way

References

1 Di Luzio N R 1959 Lipid composition of Kupffer cells Am J Physiol 196 884
2 Schoenheimer R & D Yuasa 1929 Z physiol Chem Hoppe Seyler's 180 19
3 Kimmelstiel P & E Laas 1934 Beitr path Anat 93 417
4 Day A J & J E French 1959 The synthesis and hydrolysis of cholesteryl ester by cells of the reticulo endothelial system Quart J Exptl Physiol 44 239
5 Adlersberg D & H Sobotka 1958 Pathological manifestations of abnormal cholesterol metabolism In Cholesterol Chemistry Biochemistry and Pathology R P Cook Ed Academic Press New York N Y
6 Landing B H & H S Shirkey 1957 Syndrome of recurrent infection and infiltration of viscera by pigmented lipid histiocytes Pediatrics 20 431
7 Lautsch E V G C McMillan & G L Duff 1958 Atherosclerosis in rabbits after intravenous injection of colloidal solutions A M A Arch Pathol 65 40
8 Brown H Z I Philips & B M Kagan 1952 The role of the reticuloendothelial system in vitamin A and cholesterol metabolism Metabolism 1 349
9 Blankenhorn D H D G Freiman & H C Knowles Jr 1956 Carotenoids in man The distribution of epiphasic carotenoid in atherosclerotic lesions J Clin Invest 35 1243
10 Krinsky N I D G Cornwell & J L Oncley 1958 Transport of vitamin A and carotenoids in human plasma Arch Biochem Biophys 73 233
11 Smith C W & P C Johnson 1959 The removal of intravenously injected triglycerides from the plasma of young men with arteriosclerotic heart disease Clin Research 7 60
12 Havel R J & D S Fredrickson 1956 The removal of palmitic acid 1 C^{14} labeled chylomicra from dog plasma J Clin Invest 35 1025 *See also* Cleland W W & J M Iacono 1957 Role of lipoprotein lipase in intravascular clearing of fat in human subjects Federation Proc 16 383
13 Fredrickson D S & R S Gordon Jr 1958 Transport of fatty acids Phys Rev 38 585
14 Morris B & J E French 1958 The uptake and metabolism of C^{14} labeled chylomicron fat by the isolated perfused liver of the rat Quart J Exptl Physiol 43 180
15 Hillyard L A C E Cornelius & I L Chaikoff 1959 Removal by the rat liver of palmitate 1 C^{14} bound to albumin and of palmitate 1 C and cholesterol 4 C^{14} in chylomicrons from perfusion fluid J Biol Chem 234 2240
16 Brauer R W G F Leong R F McElroy & R J Holloway 1956 Circulatory pathways in the rat liver as revealed by P^{32} chromic phosphate colloid uptake in the isolated perfused liver preparation Am J Physiol 184 593
17 Halpern B N 1959 The role and function of the reticulo endothelial system in immunological processes J Pharm and Pharmacol 11 321
18 Fredrickson D S D L McCollester R J Havel & K Ono 1958 The early steps in transport and metabolism of exogenous triglyceride and cholesterol *In Chemistry of Lipids as Related to Atherosclerosis* I H Page Ed Thomas Springfield Ill
19 French J E & B Morris 19 The removal of C labelled chylomicron fat from the circulation in rats J Physiol London 138 326
20 Feigenbaum L S O Byers & M Friedman 1954 Role of reticuloendothelial system and disposition of dietary cholesterol in various mammalian species Pr Soc Exptl Biol Med 85 530
21 Friedman M & S O Byers 19 4 The relationship of the hepatic reticuloendothelial cell (Kupffer cell) to endogenously induced cholesterol Circulation 10 491
22 Friedman M S O Byers & R H Rosenman 19 4 Demonstration of the essential role of the hepatic reticulo-endothelial cell (Kupffer cell) in the normal disposition of exogenously derived cholesterol Am J Physiol 177 77
23 Friedman M S O Byers & S St George 19 6 Detection of dietary cholesterol 4-C^{14} in the hepatic reticulo endothelial cell of the rat Am J Physiol 184 141

pression in the removal rate of these colloids as well as a possible alteration in their organ distribution should occur during the time of alimentary lipemia The colloidal carbon disappearance rate[6] was studied in 9 normal male dogs in a postabsorptive state and in 7 dogs rendered markedly lipemic by the feeding of liquified lard in the amount of 4 5 gm /kg body weight The normal dogs injected with 200 mg carbon/kg had a mean half time (t/2) of 12 2 min , the lipemic dogs had a mean intravascular half life of 9 5 min A study of the intravascular half life of radioactive colloidal gold indicated that dogs exhibiting a lipemia due either to lard or olive oil feeding had a significantly enhanced removal rate (TABLE 1) No differences were observed in the organ uptake of radiogold in the lipemic group Thus despite the myriads of chylomicra present no competitive inhibition of phagocytic function was noted and a slight enhancement in phagocytic function may well have occurred in the lipemic group

With the development[3] and improvement[7] of a technique to isolate a possible representative sample of Kupffer s and parenchymal cells a more direct method was available to determine the relative role of hepatic cells in lipid

TABLE 1

COLLOIDAL GOLD DISTRIBUTION IN NORMAL AND LIPEMIC DOGS

Group	No. animals	Half time (sec.)	Liver	Lung	Spleen
Normal	10	123	90 5	0 38	1 34
Lipemic olive oil fed	7	99 4	86 5	0 19	1 38
Lipemic lard fed	8	86 8	86 8	0 17	1 07

Values are expressed as per cent of the injected dose per total organ

metabolism *The Kupffer s and parenchymal cell distribution[7] of an I^{131} labeled* triolein emulsion was studied in 7 rats The plasma disappearance rate and organ distribution of this emulsion has been demonstrated to be similar to that of native chylomicra Approximately 40 per cent of the injected dose was localized in the liver 15 min following injection The cellular distribution was 47 per cent in the isolated parenchymal cells and 2 per cent in the Kupffer s cell population These data are indicative of a major role of the parenchymal cells in the removal of chylomicra from blood

Friedman and Byers[8] and Byers elsewhere in this monograph have reported that Kupffer s cells participate in the exclusive uptake of cholesterol containing chylomicra Their studies on isolated hepatic cells were conducted *6 and 24 hours following the oral administration of C^{14} labeled cholesterol* It is apparent that it is most difficult to determine the precise pathway of the hepatic uptake of cholesterol at such periods

The hepatic cellular distribution of a cholesterol lecithin emulsion was studied in rats at early and at frequent intervals following its intravenous administration Plasma samples obtained 15 min after the injection of the emulsion showed a mean 900 per cent increase in free cholesterol and a 72 per cent increase in phospholipid The mean half time of the phospholipid

RETICULOENDOTHELIAL INVOLVEMENT IN LIPID METABOLISM*

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Previous studies have demonstrated that the liver is not only the major organ involved in triglyceride and phospholipid metabolism but also that it participates extensively in the endogenous production and excretion of endogenous and exogenous cholesterol The liver has been defined as being composed of 61 per cent parenchymal and 33 per cent littoral cells[1] Although it is of possible importance to the etiology of lipid storage diseases and experimental atherosclerosis little is known of lipid metabolic activity at the hepatic cellular level Although evidence has been cited for the possible participation of the reticuloendothelial system (RES) and particularly of the Kupffer's cells in the normal removal of ingested fat[2] and cholesterol[3] from blood, reticuloendothelial involvement in lipid metabolism is as yet one of the uncertain functions of the macrophage system In previous studies however so much importance had been attached to the RES that it was considered an intermediary apparatus in cholesterol metabolism[4] Rothschild[5] suggested in 1914 that Kupffer s cells are intermediary and liver the regulatory organ in cholesterol metabolism inasmuch as they maintain the cholesterol equilibrium of the organism through the elimination of cholesterol in bile The demise of the concept of reticuloendothelial blockade as a technique to produce permanent functional depression of the RES has forced a reconsideration of reticuloendothelial participation in lipid metabolism

The present studies were undertaken in an attempt to evaluate quantitatively the role of hepatic parenchymal and Kupffer s cells in triglyceride and cholesterol metabolism Results of the latter studies appeared to indicate a possible role of Kupffer s cells in the biotransformation or excretion of cholesterol If this concept were correct it would be anticipated that during Kupffer s cell hyperplasia and hyperfunction increased tolerance to ingested cholesterol should develop as manifested by reduced tissue cholesterol levels of animals maintained on an excessive intake of dietary cholesterol Additional studies were therefore conducted on the relationship between Kupffer s cell activity and cholesterol metabolism particularly the development of hypercholesterolemia and cholesterosis

Results and Discussion

It has been demonstrated that with an increase in the particle number of injected colloidal materials a corresponding depression in their removal rate occurs[6] Thus it would be anticipated that if triglyceride containing chylomicra were handled in a manner similar to colloidal carbon (Gunther Wagner C 11/1431a) or colloidal gold (Aurocoloid Abbott) a significant de

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† Lederle Medical Faculty Awardee

The remaining group 9 of which received zymosan were fed an atherogenic diet similar to that employed by O Neal *et al* [19] as shown in TABLE 3

Daily mea urement were made of body weight and food intake On the

TABLE 3
COMPO ITION OF ATHEROGENIC DIET FED TO 18 RATS

Ing i t	P t g
Butter	37 0
Ca in (itamin free)	19 9
Sucrose	18 1
Alphacel (nonnutritive bulk)	9 1
Salt mix U S P XIV	7 3
Cholesterol	4 5
Thiouracil	0 3
Choline chlori le	0 2
Vitamin diet fortificati n mixture	1 8
Sodi um cholate	1 8

TABLE 4
INFLUENCE OF ZYMOSAN ON HEPATIC LIPIDS OF NORMAL AND CHOLESTEROL FED RAT

G p	N	L per body w ght	Ch l t l		N l l f t
			F	E t	
Control	9	3 7	1 82	0 71	9 14
Control and zymosan	9	6 3	2 18	0 35	6 81
Cholest ol fed	9	5 4	4 60	50 56	33 93
Choleste ol fed and zymosan	9	7 1	3 45	17 20	17 89

Lipid alues are exp ressed as milligram per gram of fresh tissue

TABLE 5
PLASMA AND SPLEEN CHOLESTEROL CONCENTRATIONS

G p	Zym	N	Pl m		Spl per body w ght	Spl	
			F	E t		F	E t
Cont ol	–	9	0 128	0 391	0 23	2 77	0 44
Cont ol	+	5	0 295	0 538	1 10	3 46	0 36
Cholest ol fed	–	9	2 64	8 72	0 24	3 79	2 33
Choleste ol fed	+	9	1 84	4 35	0 78	3 93	2 31

Pla ma alu ar expressed as milligrams per cubic cent m ter spl en values as milli grams p r gram

fifteenth day liver pla ma and spleen lipids were determined according to previou ly de cribed procedures [7] *In agreement with previous ob ervations*[17,18] zymosan admini tration increased liver and pleen weight significantly (TABLES 4 and 5) Tis ue *lipid were not significantly altered in normal rats given* zymosan Maintenance of rats on the experimental diet profoundly elevated

component of the emulsion was 3.5 min This value is similar to that reported for P^{32} labeled homologous plasma phospholipid [9] The importance of employing lipid emulsions with physiological properties has been stressed since the observations of Murray and Freeman [10]

The isolation of hepatic cells[7] and the determination of the cellular distribution of free and ester cholesterol indicated that an initial and sustained elevation occurred in the parenchymal cell free cholesterol fraction at which time the Kupffer's cell cholesterol concentration was not significantly altered (TABLE 2) The concentration of cholesterol in the isolated Kupffer's cells 60 min following its injection showed a mean 10.5 per cent elevation over control values No significant differences were observed in the ester cholesterol fraction

The results of our studies with triglyceride emulsions are basically in agreement with the observations of Murray and Freeman [10] Morris and French [11] Woerner,[1] Waddell *et al* [13] and Bailey *et al* [14] The present results support the concept[13] that free permeation of fat into the hepatic parenchymal cell

TABLE 2
HEPATIC CHOLESTEROL CONCENTRATIONS FOLLOWING INTRAVENOUS CHOLESTEROL

Time (min)	No. animal	Free Cholesterol		Ester Cholesterol	
		Kupffer's	Parenchymal	Kupffer's	Parenchymal
0	6	9.1	7.4	4.1	2.4
15	6	11.7	13.3	1.9	1.2
30	6	12.4	14.9	4.4	2.4
60	6	18.7	14.7	3.8	1.5

Values are expressed as milligrams of lipid per gram of dry defatted tissue

is the principal method of passage of lipid into the liver from the blood stream possibly *through the sinusoidal pores that have been demonstrated* [15] *Our* results question the concept of an exclusive role of Kupffer's cells in the uptake of exogenously administered triglyceride or cholesterol[8] (*see also* Byers elsewhere in this monograph) Studies on the hepatic distribution of cholesterol however suggested that Kupffer's cell might participate in either the biotransformation or excretion of cholesterol A previous discussion of this possibility has been entertained by Stambul [16] To evaluate this concept of Kupffer's cell participation in cholesterol metabolism the influence of RE hyperactivity on tissue cholesterol levels was studied in rats maintained on normal and high cholesterol high fat diets If Kupffer's cells participated in cholesterol metabolism increased tolerance to ingested cholesterol should be manifested during reticuloendothelial hyperfunction as indicated by lower tissue cholesterol level The RES was stimulated by intravenous injections of the polysaccharide yeast extract zymosan which has previously been demonstrated to produce RE hyperfunction and hyperplasia [17,18] Thirty six male rats were initially employed 9 normal rats maintained on Purina Chow served as controls and received saline while 9 others received zymosan

prevented the development and arrested the progress of atherosclerosis while stimulating phagocytosis In the zymosan treated rats we have not observed an increased cholesterol content of spleen that Constantinides observed in rabbits treated with manuronate

In view of our findings and the known mode of cholesterol excretion which in the rat is an array of bile acids the hypothesis shown graphically in FIGURE 1 can be proposed This concept delineates the possible mechanism whereby zymosan inhibits the accumulation of liver and plasma cholesterol and the possible etiology of hypercholesterolemia in the untreated rat

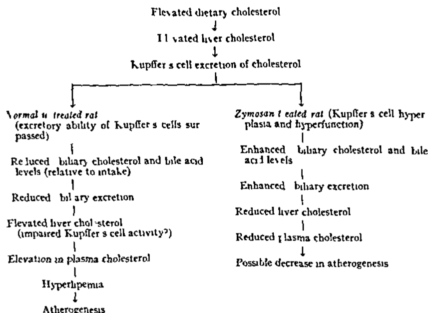


FIGURE 1 Schematic diagram of a proposed concept to delineate the possible mechanism whereby zymosan inhibits the accumulation of liver and plasma cholesterol and the possible etiology of hypercholesteremia in the untreated rat

This hypothesis is presently being evaluated In view of the role of zymosan in immunological mechanisms host resistance and the functional complexity of the RES the protective action of zymosan could conceivably involve other phases of reticuloendothelial activity The present findings however not only indicate a metabolic function of the Kupffer's cell in cholesterol metabolism but also suggest a new concept in the regulation and prevention of hypercholesterolemia hepatic lipidosis and cholesterosis through activation of the RES

Summary

During alimentary lipemia induced in dogs by the feeding of either saturated or unsaturated fats no significant impairment occurred in phagocytic function as indicated by the rate of clearance of colloidal carbon or gold

ester cholesterol fractions of liver, plasma and spleen The intravenous administration of zymosan in 10-mg amounts on alternate days to rats maintained on the atherogenic diet induced a comparable increase in liver and spleen weight Zymosan treatment markedly reduced the accumulation of free and ester cholesterol and neutral fat of liver and produced a significant decrease in plasma cholesterol Zymosan was without effect on the elevated ester cholesterol fraction of spleen

The effectiveness of zymosan in inhibiting hepatic cholesterosis at lower levels of dietary cholesterol intake was also studied Six male rats were pair fed a Purina chow diet that contained 1 per cent cholesterol and 0 5 per cent sodium cholate The rats lost a mean 9 per cent of their initial body weight In the untreated cholesterol fed rats the liver ester cholesterol concentration was markedly elevated (TABLE 6) As previously observed a significant reduction occurred in the liver ester cholesterol fraction of rats that were injected daily with zymosan in the amount of 5 mg/day beginning 1 day before the start of the feeding period

TABLE 6
EFFECT OF ZYMOSAN ON LIVER CHOLESTEROL LEVELS OF PAIR FED RATS ON A 1 PER CENT CHOLESTEROL DIET

Group	Per cent body weight		Cholesterol	
	Liver	Spleen	Free	Ester
Normal	3 5	0 16	1 90	0 32
	3 4	0 22	1 91	0 18
Cholesterol fed	2 9	0 22	3 12	17 15
	3 1	0 27	3 28	12 51
Cholesterol fed + zymosan	4 0	0 72	2 78	2 13
	3 9	0 66	2 54	1 56

Values are expressed as milligrams per gram of fresh tissue

It appears that a major determinant to elevated tissue cholesterol levels of rats maintained on high cholesterol diets is the functional status of the RES and specifically the number and activity of Kupffer s cells The limited evidence for Kupffer s cell involvement in the biliary excretion of cholesterol or its degradation products has been previously presented by Stambul[19] Further support of reticuloendothelial involvement in cholesterol metabolism is suggested by the observations that experimental hypercholesterolemia and atherosclerosis *become manifest only after the infiltration of cholesterol* in organs essentially comprising the RES and particularly the liver[20] Brown *et al*[21] have tated that the integrity of the RES is important in cholesterol metabolism The accumulation of lipid in Kupffer s cells was reported to be most marked in diabetes[22] a condition associated with impaired RE activity[23] hyperlipemia and an increased incidence of atherosclerosis The possible tendency of rabbits with less active RES to develop higher serum cholesterol levels during periods of dietary cholesterol excess has been suggested[24] Conversely Constantinides[25] reported that sulfated alginic acid

20 Anitschkow N. 1933. Experimental arteriosclerosis in animals. 271. In Arteriosclerosis: A Survey of the Problem. E. V. Cowdry, Ed. Macmillan. New York, N.Y.
21 Brown H. Z., I. Philips & B. N. Kagan. 1952. The role of the reticulo-endothelial system in vitamin A cholesterol metabolism. Metabolism 1: 343.
22 Rossle R. 1907. Ueber die Leber beim Diabetes. Verhandl. deut. Pathol. Ges. 10: 334.
23 Juhlin L. 1959. The phagocytic activity of the reticulo-endothelial system in alloxan diabetic rabbits. Acta Physiol. Scand. 45: 369.
24 Neveu T., C. Biozzi, B. Benacerraf, C. Stiffel & B. N. Halpern. 1956. Role of reticulo-endothelial system in blood clearance of cholesterol. Am. J. Physiol. 187: 269.
25 Constantinides P. & P. Saunders. 1958. Atherostatic and [illegible]phage stimulating effects of mannuronate. A.M.A. Arch. Pathol. 66: 499.

The tissue distribution of colloidal gold was unaltered in lipemic dogs Studies on the cellular distribution of intravenously administered triglyceride and cholesterol emulsions indicated that the major site of removal of these lipids is the hepatic parenchymal cell The subsequent elevation in Kupffer's cell cholesterol content appears to be indicative of a metabolic or excretory function of these cells in cholesterol metabolism In support of this concept reticuloendothelial hyperplasia and hyperfunction were induced by repeated intravenous injections of zymosan in rats maintained on normal and high cholesterol diets Rats with induced hyperplasia and hyperfunction manifested a profound lowering of liver and plasma cholesterol Spleen lipids were unaltered These studies indicate a reticuloendothelial participation in experimental hypercholesterolemia and a preventive action of zymosan

References

1 DAOUST R 1958 The cell population of liver tissue and the cytological reference base *In* Liver Function 3 R W Brauer Ed Am Inst Biol Sci Washington D C
2 JAFFE P H & S L BERMAN 1928 The relations between Kupffer cells and liver cells A M A Arch Pathol 5 1070
3 BYERS S O S ST GEORGE & M FRIEDMAN 1957 Hepatic reticulo endothelial cells as participants in the normal disposition of exogenous cholesterol in the rat Physiopathology of the reticulo endothelial system 128–147 B N Halpern Ed Thomas Springfield Ill
4 HARBITZ F 1927 Tumors of the tendon sheaths joint capsules and multiple xanthoma A M A Arch Pathol 4 507
5 ROTHSCHILD M H 1914 Ueber die Beziehungen der Leber zum Cholesterinstoffwechsel Beitr pathol Anat u allgem Pathol 60 66
6 BIOZZI G B BENACERRAF & B N HALPERN 1953 Quantitative study of the granulopectic activity of the reticulo endothelial system II A study of the kinetics of the granulopectic activity of the RES in relation to the dose of carbon injected Relationship between the weight of the organs and their activity Brit J Exptl Pathol 34 441
7 DI LUZIO N R 1959 Lipid composition of Kupffer cells Am J Physiol 196 884
8 FRIEDMAN M S O BYERS & S ST GEORGE 1956 Detection of dietary cholesterol 4 C^{14} in the hepatic reticulo endothelial cell of the rat Am J Physiol 184 141
9 ZILVERSMIT D B & J L BOLLMAN 1956 Role of the liver and intestine in the turnover of plasma phosphatides in the rat Arch Biochem Biophys 63 64
10 MURRAY R G & S FREEMAN 1951 The morphologic distribution of intravenously injected fatty chyle and artificial emulsion in rats and dogs J Lab Clin Med 38 56
11 MORRIS B & J F FRENCH 1958 The uptake and metabolism of C^{14} labelled chylomicron fat by the isolated perfused liver of the rat Quart J Exptl Physiol 43 180
12 WOERNER C A 1949 Cytological distribution of fat injected intravenously into guinea pigs Anat Record 104 61
13 WADDELL W R R P GEYER E CLARKE & F J STARE 1954 Function of the reticulo endothelial system in removal of emulsified fat from blood Am J Physiol 177 90
14 BAILEY J M G O GEY & M K GEY 1959 Utilization of serum lipids by cultured mammalian cells Proc Soc Exptl Biol Med 100 686
15 FAWCETT D W 1955 Observations on the cytology and electron microscopy of hepatic cells J Natl Cancer Inst 15 1475
16 STAMBUL J 1952 The Mechanisms of Disease 205–279 Froben New York N Y
17 KELLY L S 1958 *Proliferation of the liver reticulo endothelial system* Federation Proc 17 84
18 BENACERRAF B & M M SEBESTYEN 1957 Effect of bacterial endotoxins on the reticulo endothelial system Federation Proc 16 860
19 O NEAL R M W A THOMAS & W S HARTROFT 1959 Dietary production of myocardial infarction in rats Am J Cardiol 3 94

injection in the tail vein While the time interval between injection of radiogold and hepatectomy is not critical a 24 hour interval was used in order to permit recovery of the animal before the next operative procedure As the second step (II), partial hepatectomy (65 to 70 per cent) was done under ether anesthesia following essentially the technique of Higgins and Anderson[2] The liver specimen removed by hepatectomy was weighed homoge

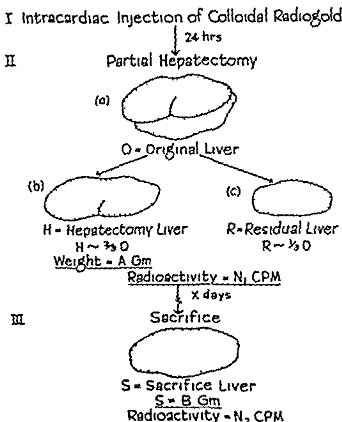


FIGURE 1 Experimental design of the isotopic-dilution method for the estimation of liver regeneration *See* text for detailed explanation

nized with formamide according to the method of Tabern *et al* [4] and subjected to liquid counting in a Geiger Muller counter This procedure yielded the radioactivity of the hepatectomy specimen designated as N_1CPM Obviously the same activity per gram was also present in the remaining liver tissue (residual liver) After hepatectomy the rats were sacrificed at time intervals ranging from 1 to 14 days (III) and their livers were removed weighed and homogenized The homogenates prepared from the entire liver in order to avoid inhomogeneities in distribution of radiocolloid in different

STUDIES ON RETICULOENDOTHELIAL FUNCTION IN RELATION TO GROWTH PROCESSES*

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Three principal functional activities of reticuloendothelial tissues phagocytic, immunological, and metabolic have formed the subject of informative presentations in this monograph One cannot help being impressed by the fact that phagocytosis represents the most commonly employed methodology for study and evaluation of reticuloendothelial function while immunological phenomena furnish at present only indirect and incomplete information about reticuloendothelial activity Metabolic processes, presumably involving the reticuloendothelial system (RES) and concerning steroids, lipids, and protein may be said just to have entered the first phase of serious investigation I admit therefore to being exceedingly vulnerable to the criticism of precociousness in attempting to correlate reticuloendothelial function with growth processes Obviously experimental approaches to this problem are by no means definitive The situation is rather comparable to that of a shipwrecked person who tries to gather suitable material for building a raft that may carry him across an uncharted body of water to a settlement or place of rescue

Having made this *apologia pro vita sua* I propose to discuss the first experimental approach in which the phagocytic activity of hepatic macrophages has been utilized as a tool for assessing a specific growth process namely, restoration of liver tissue after partial hepatectomy in the rat The essential background of the experimental design is based on the three following considerations (1) intravascularly circulating radiocolloid is removed up to 80 to 95 per cent by macrophages in the liver and remains there for at least several weeks although later redistribution may occur [1] (2) when portions of liver tissue removed by partial hepatectomy are assayed for content of radiocolloid results of these tests also establish the radiocolloid concentration in the hepatic tissue left behind in the body (the residual liver) and (3) since the liver present in an animal sacrificed at a specific time after hepatectomy is made up of the residual liver plus the regenerated liver and still contains the same amount of radiocolloid as was originally present in the liver tissue left in the body at the time of partial hepatectomy this permits calculation of the dilution of the radiocolloid by the newly formed liver tissue

FIGURE 1 illustrates the general design of the experiments for which inbred Lewis rats 2½ to 5 months old and of both sexes were used The first step (I) was intracardiac injection of colloidal radiogold into the anesthetized rat this technique was preferred to intravenous injection because it gave greater assurance for intravascular deposition of the radiocolloid than

* The work reported in this paper was supported in part by grants from the Illinois Chapter of the American Cancer Society Inc the Leukemia Research Foundation and the Evelyn Steinberg Memorial Club for Cancer Research all of Chicago Ill

of hepatectomy liver multiplied with the weight in grams of the residual liver (FIGURE 1) Thi in turn permits olution for and calculation of the unknown weight of the residual liver (step I) Since it is well known that a regenerating liver has a higher water content than a resting one the amount of solid per gram was determined in both liver portions in each experiment and correction neces ary becau e of the difference in solid were incorpo rated in step II Finally knowledge of the weights of residual and hepat ectomy liver permit calculation of the weight of the original liver thus en

TABLE 2
RESULTS OF I OTOPIC DILUTION TECHNIQUE FOR E TIMATION OF LIVER REGENERATION AFTER PARTIAL HEPATECTOMY

S f ed day f h patectomy	S	N f t	Li: P t f sol t M ± SD	Li: P t eg t M ± SD
0	M F	100 85	29 6 ± 0 9 29 7 ± 1 2	— —
1	M F	8 8	25 1 ± 1 5 26 9 ± 2 3	35 8 ± 6 5 45 9 ± 3 6
2	M F	15 13	24 5 ± 1 4 25 7 ± 2 1	37 7 ± 5 6 38 9 ± 2 6
3	M F	14 11	23 0 ± 0 9 23 7 ± 1 3	50 1 ± 6 4 59 3 ± 5 3
5	M F	11 11	25 9 ± 3 3 26 6 ± 1 8	82 1 ± 6 5 76 6 ± 8 5
7	M F	13 14	25 6 ± 1 3 25 8 ± 1 9	67 6 ± 8 9 87 7 ± 10 4
11	M F	17 16	27 1 ± 1 1 27 2 ± 1 4	96 8 ± 7 7 106 0 ± 10 8
14	M F	11 12	25 8 ± 1 5 26 0 ± 1 4	95 1 ± 11 2 103 2 ± 18 9

abling one to calculate the percentage of regeneration ob erved in a given animal at a given time by correlating weights of sacrifice and original liver (step III)

TABLE 2 summarizes data obtained by this method on 89 male and 85 fe male Lewis rats 100 male and 85 female rats served as control for deter mination of concentrations of solids in the intact liver Since these data in general compare well with those available in the literature from similar studies only a few comments appear in order (1) comparative radioa say carried out after hepatectomy and immediate acrifice yielded radioactivity values for residual and hepatectomy liver ti sues that agreed within ±2 per cent (2) attention is called to the considerable range of values within each

portions as potential source of error, were radioassayed, yielding N_1CPM as radioactivity of the liver at time of sacrifice. This activity can be assumed to be identical with the activity present in the residual liver. In other words if no new liver tissue had been formed between hepatectomy and sacrifice the radioactivity per gram of liver would be identical in residual and

TABLE 1
CALCULATION OF LIVER REGENERATION BY ISOTOPIC DILUTION TECHNIQUE

Weight of hepatectomy liver (H)	8 gm
Radioactivity (N_1) in H	800 000 cpm
Radioactivity/gm of H	100 000 cpm
Radioactivity/gm of residual liver (R)	(100 000 cpm)
Weight of sacrifice liver (S)	10 gm
Radioactivity (N_2) in S	400 000 cpm

$$\text{Radioactivity in } S = \frac{\text{Radioactivity in } H}{\text{Weight of } H} \times \text{Weight of } R$$

or

$$N_2 = \frac{N_1}{H}\text{ gm} \times R\text{ gm}$$

or

$$R = \frac{N_2}{N_1} \times H\text{ gm} \qquad (1)$$

Correction for differences in water content

s_1 = solids per gm of H = 0.25
s = solid per gm of S = 0.20

$$P\ \text{(corrected weight of residual liver)} = \left[\frac{N_2}{N_1} \times \frac{s}{s_1} \times H\text{ gm}\right] \qquad (2)$$

Calculation of per cent of liver regeneration

Original liver (O) = $R + H$

$$\text{Per cent regeneration} = \left[\frac{S}{R+H} \times 100\right] \qquad (3)$$

Example $R = \frac{400\,000}{800\,000} \times \frac{0.20}{0.25} \times 8 = 3.2$ gm

$O = R + H = 11.2$ gm

$$\text{Regeneration} = \frac{10}{11.2} \times 100 = 89.3 \text{ per cent}$$

For example 7 days posthepatectomy

sacrifice liver. Conversely the more new liver tissue has been formed the greater will be the decrease of the radioactivity of the sacrifice liver as compared with the hepatectomy specimen.

TABLE 1 presents the method for calculation of quantity of newly formed liver tissue by means of a hypothetical experiment. All values listed are results of actual determinations with the exception of the radioactivity per gram of residual liver which is inferred to be identical with the radioactivity per gram of hepatectomy liver. This assumption permits the equation of the total radioactivity in the sacrifice liver with the radioactivity per gram

while an increase of questionable significance was noted in male and female rats 8 days after operation no differences at all were detected 16 days after hepatectomy Similar results were obtained with male Wistar rats hepatic uptake was elevated 3 but not 15 days after hepatectomy and splenic uptake was increased in rats tested at both time intervals

Benacerraf and his co workers[7] in 1955 reported accelerated blood clearance of intravenously injected India ink after partial hepatectomy and interpreted these findings as reflecting chiefly the increased rate of blood flow through the reduced volume of liver In our experiments in which absorption of intraperitoneally injected colloid extended over a period of 24 hours it is not likely that such changes in blood flow could be the decisive factor Furthermore conclusive evidence for association of posthepatectomy states with increased phagocytic activity in the liver has been presented by Leong and his associates[8,9] Since these authors used the elegant technique of removing the liver at various intervals after partial hepatectomy of rats and perfusing them *in vitro* with suspensions of I^{131} labeled chromic phosphate they were able to control the rate of flow through the liver and eliminate this variable In this manner they demonstrated a threefold increase in phagocytic activity by 72 hours after hepatectomy and interestingly increased phagocytosis was observable with this method for a period of as long as 90 days after hepatectomy From the results of these independent studies using different techniques one may infer that the growth process expressed as liver restoration following partial hepatectomy is accompanied by significant increases in phagocytic function From our studies one notes that this is not confined to the liver but also involves the spleen In this connection it is worthy of note that we found moderate splenomegaly to occur after hepatectomy as shown in TABLE 4 Leong and his co-workers[9] also showed that while the total number of littoral cells in regenerating liver did not increase there was a significant increase in the relative proportion of actively phagocytic cell as judged from uptake of India ink The effect of splenectomy on liver regeneration may be also considered in this connection Higgins and Priestley[10] reported in 1932 that simultaneous splenectomy and partial hepatectomy accelerated slightly the rate of liver regeneration They noted in splenectomized rats a greater increase of histiocytes in the residual liver fragment than in rats with intact spleens Perez Tamayo and Romero[11] recently confirmed these results inasmuch as after splenectomy liver regeneration during the first 12 days was slightly accelerated Since this effect occurred to the same extent when the spleens were removed 10 days prior to or simultaneously with the hepatectomy the authors concluded that alterations in blood flow could not be responsible for enhancement of liver regeneration

The following points also may deserve comment

(1) The maximal increase in phagocytic activity after partial hepatectomy coincides with maximal mitotic activity and the peak of increase in the serum beta globulin fractions[1] This may be more than coincidental

(2) Throughout our work with hepatectomized rats as well as in other studies concerning phagocytosis in rats we found significantly higher uptakes

group as indicated by the standard deviations. This indicates the biological variability of liver regeneration which makes it desirable to have available a direct assay of regeneration, such as the one offered, in preference to the assumption that 65 to 70 per cent of the liver is removed by the technique of Higgins and Anderson.[2] (3) There seems to be a somewhat faster and greater rate of regeneration in female than in male animals. Since no detailed statistical analysis of these differences has as yet been performed, no conclusions can be drawn at present.

TABLE 3
EFFECTS OF HEPATECTOMY ON PHAGOCYTIC ACTIVITY

Strain	Days postop	Sex	Group and no. of rats		Per cent of injected dose Au per gm	
					Liver	Spleen
Lewis	3	M	S	25	1.79 }†	1.89 }†
			H	28	3.30	3.90
		F	S	16	4.26	4.01
			H	22	4.86	4.22
Lewis	8	M	S	17	1.98 }†	2.23
			H	21	3.40	2.92
		F	S	16	3.59 }§	2.74
			H	13	4.92	3.70
Lewis	16	M	S	11	1.39	2.82
			H	15	1.26	2.00
		F	S	12	2.88	4.06
			H	16	2.72	2.91
Wistar	3	M	S	24	1.51 }†	1.77 }†
			H	23	2.92	2.44
Wistar	15	M	S	17	1.71	1.55 }‡
			H	18	1.97	1.95

S, sham operated; H, hepatectomized.
† $P < 0.001$
‡ $P < 0.01$
§ $P < 0.05 > 0.01$

Turning now to studies of specific interrelations between phagocytic function and growth, reference is made first to observations concerning one of the most active nonneoplastic postnatal growth processes, namely, restoration of liver tissue after partial hepatectomy. We have studied the uptake in liver and spleen of colloidal radiogold injected intraperitoneally in rats following hepatectomy.[5,6] As shown in TABLE 3, in male Lewis rats radioactivity per gram of liver was increased significantly 3 and 8 days after hepatectomy as shown by comparison with sham-operated controls. In female rats this increase was less pronounced. Sixteen days after hepatectomy, phagocytic activity was not distinguishable in livers of experimental and sham-operated animal. Splenic uptake in hepatectomized rats was increased significantly in male but not in female Lewis rats 3 days after operation.

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St	Day po top	S	G p nd o of t		P t f j ted d se A pe gm	
					Li	Spl
Lewis	3	M	S	25	1 79 } †	1 89 } †
			H	28	3 30	3 90
		F	S	16	4 26	4 01
			H	22	4 86	4 22
Lewis	8	M	S	17	1 98 } †	2 23
			H	21	3 40	2 92
		F	S	16	3 59 } §	2 74
			H	13	4 97	3 70
Lewis	16	M	S	11	1 39	2 87
			H	15	1 26	2 00
		F	S	12	2 88	4 06
			H	16	2 72	2 91
Wistar	3	M	S	24	1 51 } †	1 77 } †
			H	23	2 92	2 44
Wistar	15	M	S	17	1 71	1 55 } ‡
			H	18	1 97	1 95

S sham operated H hepatectomized
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the increa ed phagocyto is even more On the other hand splenic phagocytosis decreased when expressed per gram of spleen while due to the pres ence of con iderable splenomegaly uptake of the entire organ was depressed to a lesser degree Studies of two other groups of investigators Halpern *et al* [9,21] in Paris France and Old and Clarke[22,23] in New York N Y employing blood clearance of intravenously injected colloid indicated that growth of certain mou e and rat tumors was associated with increa ed phagocytic activity Additional data on this subject are presented in the paper of Old

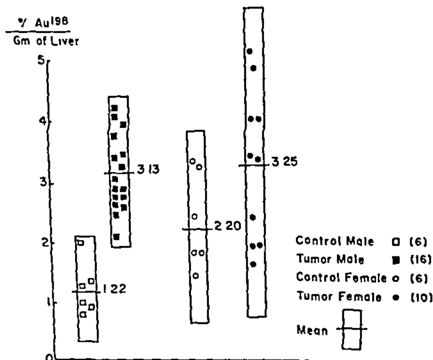


FIGURE 2 Uptake of adi g ld in l ver of control and lymphoma rats

et al el ewhere in this monograph Returning to our work it may be significant that the increa ed phagocytic activity in liver was much more pronounced and constant in rats with tran planted lymphomas than in animal with Walker carcinoma Furthermore in lymphoma bearing rats phagocy to is was not affected until tumors reached a certain size and metastatic spread had occurred Hence it would appear that either generalization of neoplastic growth or entry into the host system of products of tumor growth i responsible for bringing about changes in reticuloendothelial phagocytosis Such an assumption would fit in well with the findings of Halpern and his a sociates[20] that intravenous but not subcutaneous inoculation of Ehrlich carcinoma into mice was followed by accelerated reticuloendothelial clearance

of radiogold in liver and spleen of female than male animals. This possibly may be based on hormonal factors, especially in view of the observations of Nicol and Helmy[13] and Heller[14] on stimulation of phagocytic activity resulting from administration of estrogens.

(3) Finally, it is of interest that not only phagocytosis, but also another function involving reticuloendothelial cells, namely, immune responses were shown to be increased after partial hepatectomy. Havens *et al.*[15] reported that injection of sheep red cells immediately after hepatectomy produced significantly higher hemolysin titers in experimental than in control rats.

TABLE 4
CHANGES IN SPLENIC WEIGHTS OF RATS AFTER PARTIAL HEPATECTOMY

Group*		Strain	Sex	No. of rats	Spleen × 100 body weight M ± S.D.	P
A	Sham operated	Lewis	M	25	0.15 ± 0.03	<0.05
	Hepatectomy	Lewis	M	28	0.17 ± 0.04	>0.01
	Sham operated	Lewis	F	16	0.15 ± 0.01	<0.01
	Hepatectomy	Lewis	F	22	0.18 ± 0.03	
B	Sham operated	Lewis	M	17	0.14 ± 0.03	<0.01
	Hepatectomy	Lewis	M	21	0.17 ± 0.04	
	Sham operated	Lewis	F	16	0.19 ± 0.03	<0.001
	Hepatectomy	Lewis	F	13	0.24 ± 0.04	
C	Sham operated	Lewis	M	11	0.14 ± 0.01	>0.01
	Hepatectomy	Lewis	M	15	0.16 ± 0.02	<0.05
	Sham operated	Lewis	F	12	0.17 ± 0.02	>0.3
	Hepatectomy	Lewis	F	16	0.19 ± 0.03	
A	Sham operated	Wistar	M	24	0.18 ± 0.05	>0.05
	Hepatectomy	Wistar	M	23	0.20 ± 0.04	<0.1
C	Sham operated	Wistar	M	17	0.33 ± 0.12	>0.05
	Hepatectomy	Wistar	M	18	0.43 ± 0.20	<0.1

* A: 3 days after operation; B: 8 days after operation; C: 15–16 days after operation.

Since elsewhere in this monograph Old *et al.* dealt primarily with phagocytic activity in relation to neoplastic growth, I shall summarize only briefly some rather unexpected findings encountered in our laboratory a few years ago[16, 16a] when we tested hepatic and splenic phagocytosis in rats with transplanted lymphomas by means of radioassay of liver and spleen after intraperitoneal injection of colloidal radiogold. From some data in the literature[17] as well as from our own observations on reticuloendothelial functions in mice free of tumors but prone to tumor development,[18, 1] one might have suspected tumorous growth to be associated with depression of phagocytosis. As shown in FIGURE 2, however, there was clear-cut evidence for increased uptake of radiocolloid in the liver of tumor-bearing animals. This was the case when the percentage of injected dose was calculated per gram of liver and, since marked hepatomegaly occurred, the organ as a whole reflected

subcategory of exogenous antigenic material such as bacteria and heterologous red cells that are responsible for the phenomena of antibody formation

(2) Macromolecules that encompass a wide range of exogenous substances among them are soluble antigens with the specific ability to induce antibody formation while substances released from homografts are responsible for the homograft reaction It is in this category that one also may place the hypothetical growth factors representing endogenous products subject to phagocytosis Subsequently they may be released either in controlled amounts destroyed or converted from growth promoting into growth inhibiting factors, or vice versa

(3) The final category of autologous protein is obviously a specific instance of the action of macromolecules Although at present we have no knowledge of the chemical nature of growth factors it may be that macromolecules belong in this category The presence in reticuloendothelial cells of autologous protein has been demonstrated by Jancso[34] Hyman[35] and Gitlin[36,37] the latter investigator interpreted the presence of globulin and albumin in Kupffer's cells as evidence of catabolic processes[37]

TABLE 5
RETICULOENDOTHELIAL PHAGOCYTOSIS

Substrate	Effects
I Particulates	Storage
Antigens	Antibody formation
II Macromolecules	Storage
Soluble antigens	Antibody formation
Homograft	Homograft reaction
Growth factors	Growth regulation
III Autologous protein	Catabolism and anabolism

Although at present the hypothesis connecting reticuloendothelial function with growth processes is admittedly tenuous I shall refer in conclusion to six experimental observations that appear to be compatible with it

(1) *Regenerative growth* The increased phagocytic activity following partial hepatectomy may represent an adaptive process initiated by the release of large amounts of growth promoting substances

(2) *Wound healing* This process has been reported to be accelerated in guinea pigs injected with antireticulocytotoxic serum (ACS)[38]

(3) *Spleen and growth* Reference has been made to the slight acceleration of early stages of liver restoration observed in splenectomized animals subjected to partial hepatectomy[10,11] Increased mitotic activity following splenectomy of rats has been reported to occur in uterine deciduoma a finding thought to indicate inhibition of growth by some splenic elements[29] The same interpretation has been applied to the depression of hematopoiesis associated with hypersplenism

(4) *Neoplastic growth* Examples of increased phagocytosis in tumor hosts have been presented One must keep in mind that this phenomenon is not associated consistently with neoplasia and that it may be manifest only in

of intravenously injected colloid In general differences in species in types and stages of tumors, in colloid, and in the methodology used for assay of phagocytosis are probably important variables that may account for divergent findings recorded in the literature concerning phagocytic function in tumor hosts Nevertheless, it may be permissible to propose some tentative interpretations of the observations made by Halpern *et al* [20,21] Old and Clarke,[22,23] and in our laboratory Some stages of neoplastic growth are associated with the release of substances that affect phagocytic function in liver and other sites While hepatic macrophages show increased activity, this may not hold true for other reticuloendothelial tissues since in our experiments splenic phagocytosis was not increased and was possibly even depressed Furthermore, Old and Clarke[3] and we[24] observed depression of another reticuloendothelial function namely antibody formation at the same time that increased phagocytosis was demonstrable in tumor bearing animals

In the final and admittedly speculative portion of this presentation I offer the hypothesis that growth processes are initiated by, or associated with, release of macromolecular substances that may be designated as growth regulators and that are capable of some interaction with reticuloendothelial cells Most likely there are enhancing as well as inhibiting factors, especially in relation to self limited growth such as wound repair or restoration of liver tissue after partial hepatectomy In neoplastic growth it has been surmised for some time that a disturbed equilibrium between promoting and inhibiting growth factors may be one of the critical factors It is also likely that minimal amounts of growth promoting and growth inhibiting substances are produced more or less constantly in connection with the attrition of tissues

In one specific growth process namely liver restoration after partial hepatectomy presence of humoral growth promoting substances has been demonstrated in the form of increased mitotic activity resulting from parabiosis of intact with hepatectomized rats[25,29] as well as from injection of serum from hepatectomized animals[28,29] One may speculate that presence of these humoral growth factors may bear a relationship to the considerable changes in plasma proteins that occur in rats following partial hepatectomy specifically the consistent increase of the beta globulin fraction[12,30] Interestingly similar though less pronounced changes occur after sham operation and wound healing[1] and hence may reflect growth in general In this connection attention is called also to the recent observations of Kamrin[31] who reported striking increase in beta globulin in parabiotic rats The possible connection of these findings with reticuloendothelial function is provided by ample observations on plasma protein changes associated with disturbances of the reticuloendothelial system such as the clinical condition of macroglobulinemia of Waldenstrom[32] and experimental studies in which administration of macromolecular compounds such as methylcellulose resulted in severe dysproteinemia with increase of globulin fractions[33]

This concept and its place within the framework of the physiology of reticuloendothelial phagocytosis are indicated in TABLE 5 One may arbitrarily divide phagocytosis into three categories

(1) Particulate matter both exogenous and endogenous with the specific

8 Leong C F I I Pessotti & I W Brauer 1958 Federation Proc 17 388
9 Leong C F I I Pessotti & R W Brauer 1959 Am J Physiol 197 880
10 Hi cin (M & J T Irifstley 1932 AMA Arch Pathol 13 573
11 Lerez Tamayo R & R Lomero 1958 Lab Invest 7 248
12 Alivizatos S C A K Stern B Savich & I Lukac 1960 Proc Soc Exptl Biol Med 103 465
13 Nicol T & I D Helmy 1951 Nature 167 199
14 Heller J H 195 Endocrinology 61 235
15 Havens W P Jr M I Schlosser & J Klatchko 1956 J Immunol 76 46
16 Stern K & A Duwelius 1958 Proc Am Assoc Cancer Research 2 348
16a Stern K & A Duwelius Cancer Research 20 In press
17 Stern K *In* 3rd Intern Symposium on the RES In press
18 Stern K 1948 Proc Soc Exptl Biol Med 67 315
19 Stern K 1955 Proc Am Assoc Cancer Research 2 48
20 Halpern B N C Biozzi M Guérin C Stiffel & D Mouton 1957 Compt rend acad sci 245 760
21 Biozzi G C Stiffel B N Halpern & D Mouton 1958 Ann inst Pasteur 94 681
22 Old L J D A Clarke & M Goldsmith 1959 Proc Am Assoc Cancer Research 3 49
23 Old L J & D A Clarke 1959 Federation Proc 18 584
24 Stern K & I Davidsohn 1954 Proc Am Assoc Cancer Research 1 46
25 Christensen B G & E Jacobsen 1949 Acta Med Scand 234 103
26 Bucher N L R J F Scott & J C Aub 1950 Cancer Research 10 207 *Ibid* 1951 11 457
27 Wennecker A S & N Sussman 1951 Proc Soc Exptl Biol Med 76 683
28 Friedrich Freksa H & I G Zaki 1954 Z Naturforsch 9B 394
29 Cuno A D & G O Gay 1952 Proc Soc Exptl Biol Med 80 421
30 Caraffa G & V Rovati 1955 Boll Soc med chir Pisa 23 124
31 Kamrin B B 1958 Ann N Y Acad Sci 73(3) 848
32 Waldenstrom J 1948 Schweiz med Wochschr 78 927
33 Benko S 1958 Magyar Belorvosi Arch 11 132 *cit* 1959 Excerpta Med 12(Sect V) 471
34 Jancsó N & A Jancsó Gabor 1952 Experientia 8 465
35 Hyman C & P L Paldino 1954 Am J Physiol 179 594
36 Gitlin D J R Kitzenberg & W L Hughes 1958 Nature 181 1064
37 Gitlin D 1958 *In* Liver Function 260 W K Brauer Ed Am Inst Biol Sci Washington D C
38 Necheles H N C Jefferson C W Phillips S Goldberg S P Bralow & S Steinberg 1953 J Lab Clin Med 39 767
39 Rigler R 1958 Wien klin Wochschr 68 202
40 Loeb L 1945 The Biologic Basis of Individuality Thomas Springfield Ill
41 Simonsen M 1957 Acta Pathol Microbiol Scand 40 480
42 Billingham R F 1958 Ann N Y Acad Sci 73(3) 782
43 Siskind G W & L Thomas 1959 J Exptl Med 110 511
44 Gillman J C Gilbert & T Gillman 1948 South African J Med Sci 13 47
45 Hamburgh M 1954 Anat Record 119 409
46 Wilson J G 1955 Anat Record 123 313

some specific sites and at some stages of tumor growth as we have noted in rats with transplanted lymphomas

(5) *Somatic growth* Crossing the border line separating autologous and homologous substances, one may refer to effects on growth dependent on immunological phenomena that are mediated by reticuloendothelial tissues In this category one may place the old observation of atrophy of certain organs that frequently develops in one animal of a parabiotic pair[40] and to the recently described runt disease [41-43] a severe stunting of growth produced by the inoculation of newborn animals with immunologically competent cells of homologous origin

(6) *Embryonic growth* Teratogenic effects have been produced consistently when pregnant rats and mice were injected with trypan blue at certain stages of gestation [44-46] The exact mechanism of this action is as yet unknown It has been shown that this condition cannot be reproduced by other unrelated macromolecular compounds subject to reticuloendothelial phagocytosis and that the dye probably acts directly on the fetus Interestingly trypan blue and to a lesser extent dyes with lesser teratogenic activity were found to be taken up by epithelial cells of the yolk sac placenta [46]

None of these isolated observations by themselves justify any far reaching conclusions Taken together however they impart a degree of probability to the hypothetical assumption that normal and pathologic reticuloendothelial function may be involved in regulation of growth processes As is the case with any such hypothesis its main value is heuristic that is it provides incentives for further experimental work aimed at confirming observations already available explaining their mechanisms and testing the compatibility of new findings with the original hypothesis

Summary

An isotopic dilution technique has been presented in which phagocytosis of intravascular radiocolloid has been utilized for estimation of liver restoration in partially hepatectomized rats

Experimental data have been discussed from which it is apparent that increased reticuloendothelial phagocytosis is associated with a nonneoplastic growth process namely liver regeneration after partial hepatectomy and with some stages and forms of neoplastic growth

A hypothesis is offered concerning a possible role of reticuloendothelial tissues in regulation of growth presumably by phagocytosis of and action on humoral growth factors

References

1 Dobson F L & H B Jones 1952 Acta Med Scand **144**(Suppl 273)
2 Higgins C M & R M Anderson 1931 A M A Arch Pathol **12** 186
3 Tabern D L & T N Lahr 1954 Science **119** 739
4 Lahr T N R Olsen G I Gleason & D L Tabern 1955 J Lab Clin Med 45 66
5 Stern K & A Duwelius 1958 Federation Proc **17** 458
6 Stern K & A Duwelius 1959 Proc Soc Exptl Biol Med **100** 546
7 Benacerraf B G Biozzi A Cuendent & B N Halpern 1955 J Physiol London **128** 1

and spleen Since approximately 90 per cent of the injected carbon localizes in these organs modifications in their weight are correlated with changes in rates of carbon clearance [14]

Alterations in the phagocytic properties of the RES during the growth of the transplanted tumor Sarcoma 180 in Ha/ICR Swiss mice are illustrated in FIGURE 1 Values of K for a dose of carbon (16 mg/100 gm body weight) as well as growth of the tumor and its effect on liver spleen and body weight,

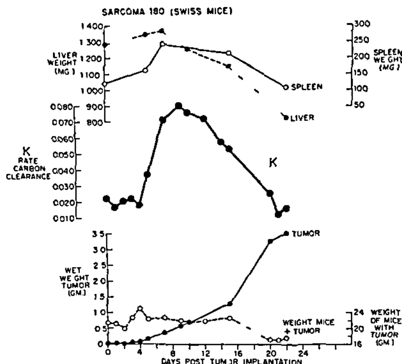


FIGURE 1 Alterations in the phagocytic capacity of the RES during growth of Sarcoma 180 in Swiss mice

are plotted in this graph each point represents an average of 5 to 10 mice During the first 4 days following tumor inoculation no alteration in phagocytic capacity from the normal K of 0.024 ± 0.008 can be detected However on the fifth day an abrupt but modest rise occurs in the rate of colloid clearance that reaches a maximum and is maintained from the seventh to the twelfth day A point bearing emphasis is the early and impressive rise in reticuloendothelial activity at a period when the tumor is still quite small With progressive growth of the tumor and deterioration of the condition of the host this marked phagocytic capacity of the RES diminishes to a normal or subnormal level By contrast the clearance rates in those animals regressing their tumors remain elevated A twofold increase in spleen

THE RETICULOENDOTHELIAL SYSTEM AND THE NEOPLASTIC PROCESS*

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A question of central importance to investigators in cancer research continues to be whether the host possesses either in a functional or latent state an active capacity to inhibit, delay or in any fashion control the development of autogenous tumors Many investigators have been intrigued by the possibility that the reticuloendothelial system (RES) may be involved in the host response to the neoplastic process, and numerous results can be found in the literature suggesting that the RES does exert some regulatory influence over the course of tumor development A comprehensive review of efforts in this area prior to 1942 has been made by Stern and Willheim [1] Many of these studies however, are difficult to interpret due to the common practice of ascribing functions to the RES that have not been demonstrated and of implying rather than measuring alterations in RE activity following experimental procedures More recently the enhanced phagocytic capacity of the RES during the growth of various transplanted tumors has been established [2-4] In addition certain agents active on the RES have been found to alter the development of a number of experimental tumors Erie Fast Rubine a sulfonated azo dye [5] Thorotrast [6] zymosan [7,8] and bacillus Calmette Guerin (BCG) infection [9-11] appear to inhibit the growth of a group of transplanted tumors in the mouse and rat Erie Fast Rubine [5] and zymosan [12] have been reported to delay the appearance of carcinogen induced sarcomas and Thorotrast and colloidal iron oxide [13] have been observed to inhibit hepatoma formation by carcinogenic azo dyes

In general we have employed two approaches to an understanding of the relationship of the RES to experimental tumor growth (1) determination of the functional activity of the RES in terms of phagocytic capacity during the growth of various transplanted carcinogen induced viral and spontaneous tumors in mice and (2) an attempt to alter the growth and lethality of tumors by agents known to produce reticuloendothelial hyperplasia

The phagocytic capacity of the RES has been determined by the clearance of a standardized preparation of carbon of homogeneous particle size [14] Under these conditions the rates of clearance (K) follow an exponential function of time according to the equation $K = [(\log C_1 - \log C_2)/(T_2 - T_1)]$ where C_1 and C_2 represent carbon concentration in the blood at times T_1 and T_2 Provided a sufficient amount of carbon is injected to challenge all available reticuloendothelial cells the value K is a measure more specifically of the phagocytic capacity of the reticuloendothelial elements of the liver

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During the growth of this tumor modest enlargement of both liver and spleen occurs As with Sarcoma 180 the maximal activation of the RES occurs before the tumor has reached an appreciable size

With the S180 ascites tumor even more striking alterations occur in reticuloendothelial function (FIGURE 4) Here the correlation between increase in liver and spleen weight and increased phagocytic capacity of the RES is more evident The clearance rates reach a maximal level by the fifth day a progressive diminution in phagocytic activity then occurs over the next several days The spleen increases approximately threefold in size whereas

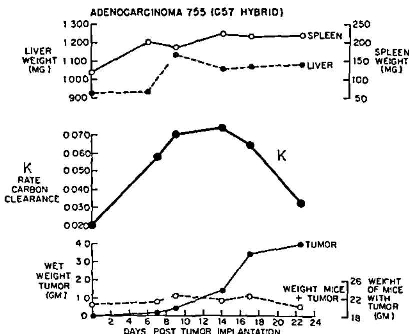


FIGURE 3 Alterations in the phagocytic capacity of the RES during growth of Carcinoma 755 in C57BL hybrid mice

the liver weight increases from an average normal value of 1 2 to 1 65 gm during the early phases of ascites formation It should be noted that terminally there is a significant depression in colloid clearance associated with a return to normal liver and spleen size

The effect of 2 variants S16 and F18 of the Ehrlich ascites tumor on the rates of carbon clearance and liver and spleen weights is represented in FIGURE 5 The parent tumor was received originally from George Klein of the Karolinska Institut Stockholm Sweden in 1950 and has been passed in Swiss mice by 2 investigators at our institute * Whereas F18 causes no al

* S16 and F18 represent the sixteenth and eighteenth transplant generation respectively in our laboratory of the S line obtained from Kanematsu Sugiura and the F line obtained from Charlotte Friend of the Ehrlich ascites tumor

weight and essentially no increase in liver weight occur during the period of maximal reticuloendothelial activity but both the liver and spleen undergo diminution in size during the terminal phase of tumor growth

Individual rates of carbon clearance were determined in a group of animals inoculated with Sarcoma 180 (S180) 9 to 11 days previously and then followed for survival (FIGURE 2) The widespread difference in reticuloendothelial response to the growing tumor is evident and the question naturally arises as to whether there is any correlation between the degree of reticuloendothelial stimulation and ultimate survival of the host in terms of tumor regression However regression of tumors was observed in animal with both marked reticuloendothelial activation as well as animals with only a slight elevation above normal clearance rates

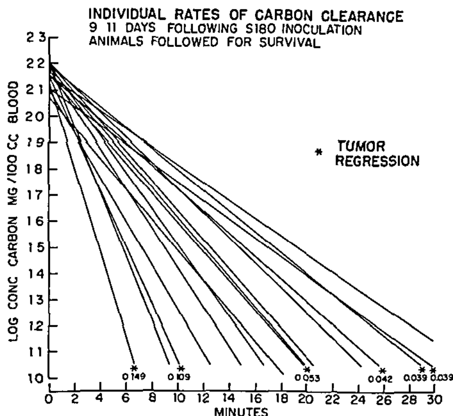


FIGURE 2 Variation in rates of carbon clearance among Swiss mice bearing Sarcoma 180

The same general host response in terms of phagocytic activity can be seen during the growth of transplantable Adenocarcinoma 755 in a C57BL hybrid mouse* (FIGURE 3) The rate of colloid clearance increases during the early phase of tumor growth reaches a maximum that is maintained for a period and then falls to normal levels prior to the death of the animal

* Produced by J J Bittner University of Minnesota Minneapolis Minn

Data concerning the activity of the RES in animals bearing spontaneous tumors are illustrated in TABLE 1 For this study the rates of clearance in old Swiss breeders with spontaneous mammary tumors were compared to rates in breeders of similar age but tumor free The average *K of 0 023* for the tumor bearers is somewhat above the *K* of 0 015 ± 0 004 for the controls The liver spleen and frequently the lymph nodes are appreciably enlarged in many of these tumor bearers and attempts are currently under

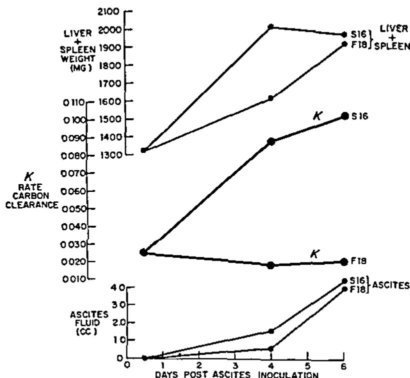


FIGURE 5 Comparison of 2 variants F18 and S16 of the Ehrlich ascites tumor on the phagocytic activity of the RES

way to determine the reason for alterations in these particular organs Since spontaneous tumors appear in older animals and only a minimal elevation of clearance rates in response to the tumor is observed the capacity of the RES of older animals to respond to stimuli that provoke reticuloendothelial hyperplasia in younger mice was studied Eighteen to 20 days following BCG infection young Swiss mice develop rates of clearance that are increased 4 to 15 times above normal The phagocytic capacity in old tumor free and tumor bearing mice following BCG infection is also increased but to a lesser extent than in younger animals It should be stressed however that ani

teration in rates, growth of S16 is associated with a considerable hyperactivity of the RES By day 6 when both S16 and F18 have caused essentially similar enlargement of the liver and spleen this difference in host response to the inoculated cells is still maintained Attempts to find bacterial contamination in either tumor have been unsuccessful

With a first generation transplant of cells from a spontaneous AKR leukemia into isologous AKR mice we have attempted to examine a trans

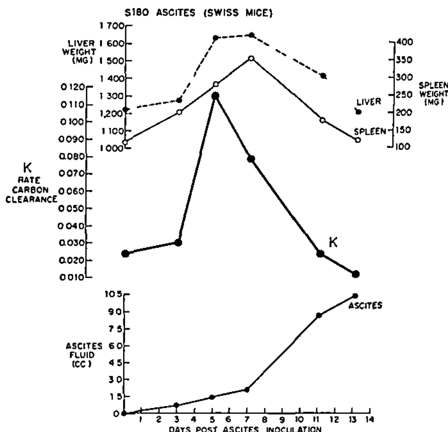


FIGURE 4 Alterations in the phagocytic capacity of the RES during growth of Sarcoma 180 ascites in Swiss mice

plantable system related as closely as possible to the spontaneous disease Young animals inoculated with leukemic tissue survive approximately 15 to 20 days and develop marked enlargement of the liver spleen and lymph nodes In the experiment represented in FIGURE 6 a very slight elevation in carbon clearance could be detected at 4 or 10 days following inoculation of a suspension of leukemic cell (average K values control AKR K = 0 012 4 days K = 0 017 and 10 days K = 0 019) A point of interest is the lower rates of carbon clearance in normal AKR mice as compared to random bred Swiss mice

infected 9 month-old Swiss mice can be accounted for partly by the smaller liver and spleen weights relative to body weight in these older animals. However when K is corrected for this factor it is still apparent that older mice have both lower basal function and a restricted capacity to respond to BCG infection.

The activation of the RES as measured by colloid clearance during the growth of various transplanted tumors appears most closely related to the foreignness of the inoculated tissue since the highest stimulation occurs with homologous tumors in the random bred Swiss mouse whereas only a questionable response was elicited by a first generation transplant of an isologous leukemia. *The heightened phagocytic activity in response to inoculated* Sarcoma 180 or Carcinoma 755 at a time when the tumor is barely palpable would tend to rule out nonspecific stimulation by products of hemorrhage and necrosis *from* the developing tumor. The fact that spontaneous tumors of Swiss mice frequently show extensive central necrosis and masses of hemolyzed blood while hosts bearing these tumors do not show a high degree

TABLE 2
RATE OF CARBON CLEARANCE IN YOUNG AND OLD VIRGIN SWISS MICE

	Age (months)	*K* (Rate of carbon clearance)	L (gm.)	Spl (gm.)	Body weight (gm.)
Controls	2-3 9	0.021 0.014	1.403 1.514	0.196 0.159	28.1 35.6
BCG	2-3 9	0.128 0.060	2.015 2.485	0.695 0.814	5.6 37.5

Five mice per group

of reticuloendothelial activation lend support to this assumption. The increased phagocytic activity may reflect participation by the RES in the general homograft reaction of the host to these transplanted tumors. That the effectiveness of this response is not usually evident in terms of tumor regression is due to the enormous growth potential of the tumor although it is possible that the tumor possesses an inherent capacity that renders it relatively more resistant to rejection by the host. The significance of the slightly faster clearance rates during growth of spontaneous mammary adenocarcinomas in Swiss mice cannot be evaluated at present. We have also seen a slight but variable (up to twofold) average increase in rates of clearance in a study of a limited number of animals with the following tumors: Carcinoma 755 grown in the homologous C57BL host, first generation transplant of a C3H mammary adenocarcinoma in isologous mice, spontaneous AKR leukemia, and sarcoma induced in Swiss mice by 1.5 mg. methylcholanthrene administered intramuscularly. No alteration in clearance rates could be detected during the time prior to tumor development in animals injected with methylcholanthrene. Careful study of a greater number of animals at multiple time intervals following tumor inoculation or appearance will be necessary to establish whether first generation transplants, spontaneous or carcinogen induced tumors con

mals with tumors are stimulated by the infectious process to about the same degree as tumor free mice of similar age. An experiment to explore the difference in reticuloendothelial function in old and young Swiss mice is represented in TABLE 2. The lower rates of clearance in both control and BCG-

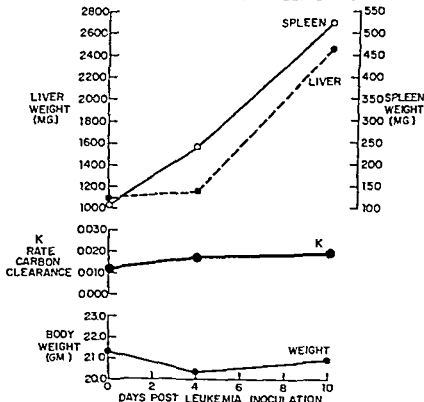


FIGURE 6. Phagocytic activity of the RES during growth of a first generation transplant of an isologous leukemia.

TABLE 1

THE EFFECT OF SPONTANEOUS MAMMARY CARCINOMA OF SWISS MICE (BREEDERS) [illegible] 12 MONTHS OLD ON THE BASAL PHAGOCYTIC ACTIVITY OF THE RES AND ON THE CAPACITY OF THE RES OF SUCH ANIMALS TO RESPOND TO INFECTION WITH BCG

(Tumor free Controls Are Breeders of Similar Age)

		No. of mice	K Rate of carbon clearance	Liver gm	Spleen gm	Body weight (gm)	Tumor weight gm
Control	tumor free	10	0.015	[illegible]	0.[illegible]4	40.1	—
	tumor	11	0.0[illegible]3	[illegible]	0.54	43.0	3
BCG	tumor free	[illegible]	0.068	3.49	0.540	41.1	—
	tumor		0.0[illegible]8	4.003	0.[illegible]3	4[illegible].4	4.1

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The activation of the RES as measured by colloid clearance during the growth of various transplanted tumors appears most closely related to the foreignness of the inoculated tissue since the highest stimulation occurs with homologous tumors in the random bred Swiss mouse whereas only a questionable response was elicited by a first generation transplant of an isologous leukemia. The heightened phagocytic activity in response to inoculated Sarcoma 180 or Carcinoma 755 at a time when the tumor is barely palpable would tend to rule out nonspecific stimulation by products of hemorrhage and necrosis from the developing tumor. The fact that spontaneous tumors of Swiss mice frequently show extensive central necrosis and masses of hemolyzed blood while hosts bearing these tumors do not show a high degree

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sistently induce enhanced phagocytic activity of the RES. Whether this activation is in response to some unique property of the tumor or is secondary to the necrosis, hemorrhage and infection commonly associated with progressive tumor growth also must be determined.

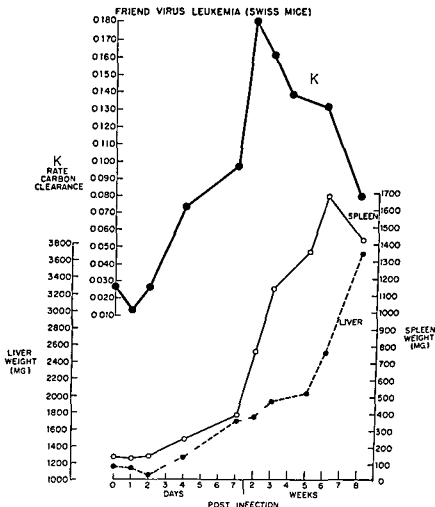


FIGURE 7. Alterations in the phagocytic capacity of the RES during development of the Friend virus leukemia in Swiss mice.

Alterations in the phagocytic capacity of the RES accompanying development of a leukemia induced by the Friend virus[16] have been among the most remarkable that we have observed (FIGURE 7). Within 24 hours following intraperitoneal inoculation of infected spleen homogenate there is a consistent depression in the rate of colloid clearance from an average normal rate in this group of mice of $K = 0.026$ to $K = 0.013$. This reduction of phagocytic capacity is not observed in mice injected with homogenate prepared from normal

mouse spleens Forty eight hours following infection the rates return to normal and by the fourth day they are elevated and continue to increase until the second week when a maximum rate of colloid clearance is reached A slow but progressive fall in clearance rates occurs during the subsequent weeks but even as late as 8 weeks following infection phagocytic activity is consistently elevated During the phase of depressed carbon clearance 24 hours following inoculation of infected spleen no alteration in liver or spleen weight could be detected From the third to the seventh day the spleen and liver undergo a progressive increase in size Following this the spleen increases enormously whereas hepatomegaly is more modest until the advanced stage of the leukemia when the liver also undergoes massive enlargement The decrease in carbon clearance during the latter phases of the leukemic process most probably reflects extensive infiltration of the liver by leukemic cell [16,17] Grossly and microscopically the organ primarily responsible for the increased rates of uptake of the injected colloid is the liver since the spleen contains only occasional cells with carbon The localization of carbon is a consequence of the very rapid rates of clearance observed in these animals and does not give indication as to the phagocytic potential of the spleen Sections of livers from infected mice reveal a prominent increase in the number of carbon containing cells lining the widely dilated sinusoids Each reticuloendothelial cell has only a fraction of the carbon seen in normal Kupffer's cells since the fixed amount of injected carbon is now distributed among an increased population of actively phagocytic cells No carbon has been seen in the characteristic leukemic reticulum cells widely infiltrating the liver and spleen We have observed the presence of round basophilic cytoplasmic masses in a small number of hepatic reticuloendothelial cells (FIGURE 8) These masses are characteristically found singly have a pale homogeneous center and are almost invariably associated with carbon particles in cells having the typical morphology of Kupffer's elements They are however clearly evident in sections of livers of infected animals receiving no carbon

In view of the enhanced phagocytic potential of these leukemic animals we decided to investigate other host characteristics that have been thought to involve in some way the reticuloendothelial system such as response to bacterial challenge ability to form antibodies and endotoxin sensitivity (TABLE 3) In general the response of the leukemic animals during the second and third weeks of infection is in contrast to that seen in animals with potentiated reticuloendothelial activity induced by other means Lipopolysaccharides [18,19] zymosan [19] and infection with BCG [20] all of which cause profound reticuloendothelial stimulation enhance resistance to bacterial challenge [1,23] and increase the capacity of the host to form antibodies [24,6] In addition BCG infection and zymosan increase the sensitivity of the animals to endotoxin [27,29] The findings of a normal response to *Klebsiella pneumoniae* challenge and *Escherichia coli* endotoxin as well as depressed hemolysin titers are opposite to what one might expect in animals with such sharply increased reticuloendothelial function Thus even though hyperplasia of reticuloendothelial cells with phagocytic potential has occurred other functional properties of these cells in particular and the RES in general have not been altered correspondingly

The findings of (1) significant depression of carbon clearance 24 hours following initiation of the infection and (2) marked and progressive stimulation of phagocytic capacity during the early phase of the disease as well as persistent reticuloendothelial stimulation throughout the course of the leukemia tend to fo



FIGURE 8. Kupffer's cell containing prominent cytoplasmic mass (*arrow*) and aggregates of carbon in the liver of a Swiss mouse with Friend virus leukemia. Hematoxylin and eosin stain. ×1000 (approx.)

TABLE 3

RESPONSE OF SWISS MICE WITH FRIEND VIRUS LEUKEMIA TO BACTERIAL CHALLENGE, ENDOTOXIN, AND SHEEP RED BLOOD CELLS DURING THE PHASE OF MAXIMAL PHAGOCYTIC ACTIVITY

Unaltered
1. Bacterial challenge with *Klebsiella pneumoniae*
2. Endotoxin sensitivity (*Escherichia coli*)
Altered
1. Decreased hemolysin formation (sheep red blood cells)

cus attention on the mature reticuloendothelial cell as a possible site for initial virus localization and propagation The spleen with its large complement of phagocytic cells might be expected to show the earliest effect of infection but since splenectomy has been shown not to alter basically the course or nature of the disease[17] the virus undoubtedly finds suitable environment for replication in other cells possibly the Kupffer elements of the liver and other reticuloendothelial cells distributed throughout the body The relationship of the hyperplasia of reticuloendothelial elements to the apparently nonphagocytic leukemic reticulum cell found infiltrating the various organs and the abnormal mononuclear cells in the peripheral blood is at present unknown Whether this represents separate responses of various cell types to the Friend agent or the disease state induced by this agent or alternatively whether a virus induced proliferation of reticuloendothelial cells occurring throughout the course of this disease leads to the formation of cells with neoplastic potential but lacking phagocytic capacity are but two of a number of intriguing possibilities

Since growth of various transplanted and spontaneous tumors is associated with activation of the RES to a variable extent an attempt to alter the development of experimental tumors by agents possessing a common capacity to induce reticuloendothelial hyperplasia has been made In these efforts we have found BCG infection and various preparations of zymosan to be most useful Endotoxins from a variety of bacterial sources have shown only suggestive activity in enhancing resistance to tumor growth

Figure 9 illustrates the effect of BCG infection induced by intravenous injection of the live organism on clearance rates and on the weight of the liver and spleen Neither morbidity nor mortality attributable to this infection was observed despite extensive proliferation of granulomatous lesions in the liver and spleen The peak in phagocytic activity associated with marked enlargement of the liver and spleen occurs fourteen to eighteen days following initiation of the BCG infection As late as four months following infection these organs are significantly larger than those of controls

The growth of Sarcoma 180 in normal Swiss mice is usually associated with an 85 to 90 per cent mortality in 2 to 5 weeks The remainder of the animals undergo a spontaneous regression of their tumors Animals inoculated with Sarcoma 180 seven days or longer following BCG infection appear to be highly resistant to tumor growth (Table 4) If the tumor and the infection are initiated within one day of each other no protection is observed Concerning this point similar results have been obtained with zymosan Intravenous injections of 1 mg zymosan one week prior to Sarcoma 180 inoculation yields better protection than zymosan injected on the day of tumor challenge The central importance of host factors in mediation of the protective effect of BCG is evident from experiments we have performed with growth of Sarcoma 180 in C57BL hybrid mice Fourteen days following BCG inoculation at a time when Swiss mice were found to be completely resistant to Sarcoma 180 growth infected C57BL hybrids were as susceptible as controls to progressive growth of the tumor The fact that C57BL hybrids do not attain as high a degree of reticuloendothelial activation as Swiss mice following BCG infection may be of importance in explaining the difference in the reaction to tumor growth in these two hosts

FIGURE 10 represents the growth of the Ehrlich ascites tumor in control and BCG infected Swiss mice Under these conditions BCG infection significantly prolongs survival time The average survival in controls was 14 days, whereas

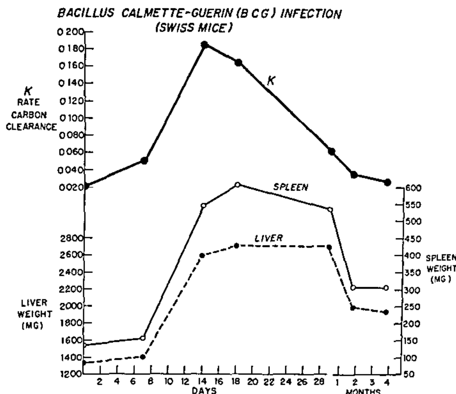


FIGURE 9 Alterations in the phagocytic activity of the RES and liver and spleen weights during the course of BCG infection in Swiss mice

TABLE 4
MORTALITY FOLLOWING IMPLANTATION OF SARCOMA 180 IN SWISS MICE AT VARIOUS INTERVALS AFTER BCG INFECTION

	D ys b tw BCG i fect d tum c l t					
Controls 68/ 9	1 13/15	7 3/12	14 0/12	19 9/30	25 0/8	67 0/9

Mortality/number per group

the BCG infected animals lived 27 days on an average Similar but somewhat less protection was obtained with zymo an injected prior to ascites inoculation

An appreciable retardation in the growth of Carcinoma 755 in the C57BL hybrid and a significant increase in time of survival are evident in the BCG infected animals (TABLE 5) The effect of BCG infection on the growth of the

same tumor in isologous C57BL mice is similar but slightly less marked (FIGURE 11) The growth of the tumor is inhibited and the animals survive longer than the controls The difference in BCC effect in these two hosts is probably related to the greater genetic foreignness of this tumor in C57BL hybrids than in the isologous C57BL animals

We have al o studied the growth of a first generation transplant of a C3H adenocarcinoma in isologous hosts No alteration in either tumor growth or

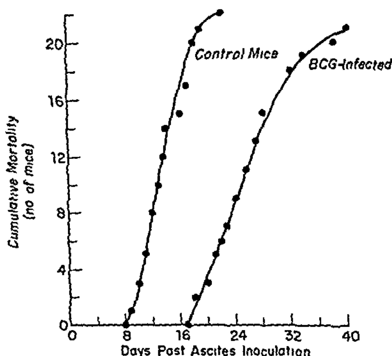


FIGURE 10 Mortality of control and BCG infected Swiss mice following inoculation of Ehrlich ascites tumor Infection with BCG initiat d 11 or 13 days prior to ascites inoculation

TABLE 5
THE EFFECT OF BCG INFECTION ON THE GROWTH OF CARCINOMA 755 IMPLANTED IN C57BL HYBRID MICE

Day	A tumor d mete n (cm)		P t mo tal ty BCG/ or t l
	13 C t l	10 BCG f t d	
25	2 04	0 82	0/0
33	2 68	1 21	0/23
48	3 56	2 39	0/92
66	—	2 90	60/100

time of death occurred in BCG infected mice Two explanations may be offered to account for the difference in isologous tumor growth in C57BL and C3H mice

(1) The Ca 755 may have been altered genetically during its multiple passages and therefore unlike the C3H first generation transplant may present antigens foreign to the host

(2) The response of the host animal to BCG infection as expressed by tumor retardation may differ considerably It has been reported that the C3H mouse is immunologically inferior to the C57BL animal in its capacity to form hemagglutinins[30] and antibodies to tetanus toxoid[31] In addition we have observed that C3H mice respond to BCG with far less increase in spleen weight than do C57BL animals To explore these possibilities further we are studying isol

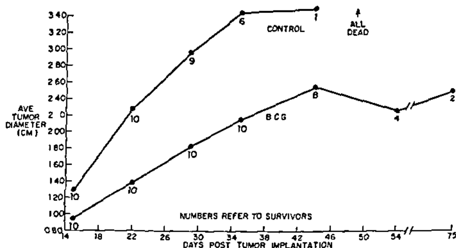


FIGURE 11 Effect of BCG infection on growth of Carcinoma 755 in isologous C57BL mice

ogous tumor growth in hosts with varying ability to form antibody and to respond to reticuloendothelial stimulation

The two agents BCG infection and zymosan which we have found most useful in protecting against tumor challenge have in addition to their ability to induce hyperplasia of the RES[19, 20] the capacity to enhance the host response to antigenic stimulation[5, 6] and to protect against challenge with various bacteria[21-23] As with bacterial challenge effective protection is afforded best when these agents are given prior to tumor inoculation However it appears that conditions successful for enhancement of resistance to bacterial infection are insufficient to protect against tumors even though the lethality of infections is usually measured in days whereas the tumor requires several weeks to kill Whether different mechanisms are involved or whether the tumor merely represents a more formidable challenge is not clear One possibility that cannot be excluded at present is that zymosan BCG and certain transplanted tumors

possess common antigenic components so that antibodies formed in response to the microbial products cross react with these tumors A more likely explanation to us would be that protection against bacterial infection following endotoxin zymosan or BCG represents an increased efficiency of individual phagocytes and increased numbers of phagocytes to ingest and destroy bacteria as well as possible alterations in non specific opsonic factor [5, 22] whereas resistance to tumor challenge involves an enhanced capacity of the host to respond by an accelerated or heightened homograft reaction The tumor protective action of zymosan or BCG infection thus could be visualized as being due to a proliferation of stem cells that have the capacity to differentiate into a broad spectrum of cell types including those involved in antibody formation and in the process of inflammation The central importance of the RES in these reactions however cannot be accepted without reservation since these agents most certainly produce wide-spread physiological alterations any one of which may play some role Nevertheless the proliferative reaction of the RES and to a lesser extent its phagocytic capacity as measured by carbon clearance appear to have significant value in predicting the efficacy of an agent in enhancing tumor resistance

Experiments are in progress to investigate the capacity of BCG and zymosan to affect the growth of carcinogen induced and spontaneous tumors One difficulty in attempting to modify the established tumor is that the tumor reaches considerable proportions before reticuloendothelial hyperplasia can be induced and maintained for any period Moreover stimuli that provoke reticuloendothelial proliferation are generally less well tolerated by hosts bearing tumors than by tumor free mice We therefore feel that a more fruitful approach may be found by studying the effect of BCG zymosan and other agents administered at various intervals during early or adult life prior to the development of carcinogen induced or spontaneous tumors

Summary

The functional capacity of the RES as determined by carbon clearance was studied during the growth of various transplanted and spontaneous mouse tumors Tumors that have been repeatedly transplanted such as Sarcoma 180 Carcinoma 755 Ehrlich carcinoma ascites and Sarcoma 180 ascites induce characteristic alteration in reticuloendothelial activity During the early phase of tumor development clearance rates are maximally elevated and remain increased throughout the period of rapid tumor growth With progressive deterioration of the tumor bearing host reticuloendothelial activity returns to normal or below normal levels A slight elevation in clearance rates has been observed during the growth of spontaneous mammary tumors in Swiss mice Striking reticuloendothelial hyperplasia following a brief phase of depressed carbon clearance accompanies development of a reticulum cell leukemia induced by the Friend virus

In addition we have investigated the effect of treatment with agents such as zymosan BCG and other products of microorganisms on the growth and lethality of various experimental tumor These agents in addition to their ability to induce reticuloendothelial hyperplasia enhance the capacity of the

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